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* * * * * * * * * *  Welcome to STN International  * * * * * * * * * *

 NEWS  1          Web Page URLs for STN Seminar Schedule - N. America
 NEWS  2          "Ask CAS" for self-help around the clock
 NEWS  3  May 12  EXTEND option available in structure searching
 NEWS  4  May 12  Polymer links for the POLYLINK command completed in REGISTRY
 NEWS  5  May 27  New UPM (Update Code Maximum) field for more efficient patent
                  SDIs in CAplus
 NEWS  6  May 27  CAplus super roles and document types searchable in REGISTRY
 NEWS  7  Jun 22  STN Patent Forums to be held July 19-22, 2004
 NEWS  8  Jun 28  Additional enzyme-catalyzed reactions added to CASREACT
 NEWS  9  Jun 28  ANTE, AQUALINE, BIOENG, CIVILENG, ENVIROENG, MECHENG,
                  and WATER from CSA now available on STN(R)

 NEWS EXPRESS  MARCH 31 CURRENT WINDOWS VERSION IS V7.00A, CURRENT
               MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),
               AND CURRENT DISCOVER FILE IS DATED 26 APRIL 2004
 NEWS HOURS    STN Operating Hours Plus Help Desk Availability
 NEWS INTER    General Internet Information
 NEWS LOGIN    Welcome Banner and News Items
 NEWS PHONE    Direct Dial and Telecommunication Network Access to STN
 NEWS WWW      CAS World Wide Web Site (general information)

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FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004

=> file uspatful
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
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FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004
CA INDEXING COPYRIGHT (C) 2004 AMERICAN CHEMICAL SOCIETY (ACS)

FILE COVERS 1971 TO PATENT PUBLICATION DATE: 8 Jul 2004 (20040708/PD)
FILE LAST UPDATED: 8 Jul 2004 (20040708/ED)
HIGHEST GRANTED PATENT NUMBER: US6760918
HIGHEST APPLICATION PUBLICATION NUMBER: US2004133957
CA INDEXING IS CURRENT THROUGH 8 Jul 2004 (20040708/UPCA)
ISSUE CLASS FIELDS (/INCL) CURRENT THROUGH: 8 Jul 2004 (20040708/PD)
REVISED CLASS FIELDS (/NCL) LAST RELOADED: Apr 2004
USPTO MANUAL OF CLASSIFICATIONS THESAURUS ISSUE DATE: Apr 2004

>>>  USPAT2 is now available.  USPATFULL contains full text of the     <<<
>>>  original, i.e., the earliest published granted patents or         <<<
>>>  applications.  USPAT2 contains full text of the latest US         <<<
>>>  publications, starting in 2001, for the inventions covered in     <<<
>>>  USPATFULL.  A USPATFULL record contains not only the original     <<<
>>>  published document but also a list of any subsequent              <<<
>>>  publications.  The publication number, patent kind code, and      <<<
>>>  publication date for all the US publications for an invention     <<<
>>>  are displayed in the PI (Patent Information) field of USPATFULL   <<<
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>>>  records and may be searched in standard search fields, e.g., /AU,  <<<
>>>  /PK, etc.                                                          <<<

>>>  USPATFULL and USPAT2 can be accessed and searched together         <<<
>>>  through the new cluster USPATALL.  Type FILE USPATALL to           <<<
>>>  enter this cluster.                                                <<<
>>>                                                                     <<<
>>>  Use USPATALL when searching terms such as patent assignees,        <<<
>>>  classifications, or claims, that may potentially change from      <<<
>>>  the earliest to the latest publication.                            <<<

This file contains CAS Registry Numbers for easy and accurate
substance identification.

=> s (HIV or human immunodeficiency virus)
         31752 HIV
        394447 HUMAN
         18315 IMMUNODEFICIENCY
         75322 VIRUS
         13121 HUMAN IMMUNODEFICIENCY VIRUS
                 (HUMAN(W)IMMUNODEFICIENCY(W)VIRUS)
L1       33450 (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)

=> s l1 and (aluminum hydroxide)
        540846 ALUMINUM
        279857 HYDROXIDE
         22295 ALUMINUM HYDROXIDE
                 (ALUMINUM(W)HYDROXIDE)
L2        3069 L1 AND (ALUMINUM HYDROXIDE)

=> s l1 and (unilamellar)
          5574 UNILAMELLAR
L3        1785 L1 AND (UNILAMELLAR)

=> s l3 and liposome?
         42761 LIPOSOME?
L4        1772 L3 AND LIPOSOME?

=> s l4 and (phosphatidylcholine)
          8350 PHOSPHATIDYLCHOLINE
L5         434 L4 AND (PHOSPHATIDYLCHOLINE)

=> s l5 and cholesterol
         36219 CHOLESTEROL
L6         402 L5 AND CHOLESTEROL

=> s l6 and mucosal
         14912 MUCOSAL
L7         107 L6 AND MUCOSAL

=> s l7 and oral
        130147 ORAL
L8          83 L7 AND ORAL

=> s l8 and ay<2000
       2994935 AY<2000
L9          22 L8 AND AY<2000

=> d l9,cbib,1-22

L9   ANSWER 1 OF 22  USPATFULL on STN
2003:268067 Particulate delivery systems and methods of use.
    Bot, Adrian I., San Diego, CA, United States
    Tarara, Thomas E., San Diego, CA, United States
    Weers, Jeffry G., San Diego, CA, United States
    Kabalnov, Alexev, Corvalis, OR, United States
```

Dellamary, Luis A., San Marcos, CA, United States
Nektar Therapeutics, San Carlos, CA, United States (U.S. corporation)
US 6630169 B1 20031007
WO 2000000215 20000106
APPLICATION: US 2000-720536 20001222 (9) <--
WO 1999-US6855 19990331 <--
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 2 OF 22 USPATFULL on STN
2003:228317 Immunostimulatory polynucleotide/immunomodulatory molecule conjugates.
Carson, Dennis A., Del Mar, CA, United States
Raz, Eyal, Del Mar, CA, United States
Roman, Mark, San Diego, CA, United States
The Regents of the University of California, Oakland, CA, United States (U.S. corporation)
US 6610661 B1 20030826
WO 9816247 19980423
APPLICATION: US 2000-308036 20000216 (9) <--
WO 1997-US19004 19971009 <--
PRIORITY: US 1996-28118P 19961011 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 3 OF 22 USPATFULL on STN
2003:203307 Therapeutic uses of keratinocyte growth factor-2.
Jimenez, Pablo, Ellicott, MD, United States
Rampy, Mark A., Montgomery Village, MD, United States
Mendrick, Donna, Mount Airy, MD, United States
Russell, Deborah, Laytonsville, MD, United States
Louie, Arthur, Potomac, MD, United States
Human Genome Sciences, Inc., Rockville, MD, United States (U.S. corporation)
US 6599879 B1 20030729
APPLICATION: US 1999-248998 19990212 (9) <--
PRIORITY: US 1998-114387P 19981230 (60)
US 1998-74585P 19980213 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 4 OF 22 USPATFULL on STN
2003:120049 TUMOR NECROSIS FACTOR RECEPTOR RELATED GENE 12 POLYPEPTIDES.
NI, JIAN, ROCKVILLE, MD, UNITED STATES
RUBEN, STEVEN M., OLNEY, MD, UNITED STATES
US 2003082532 A1 20030501
APPLICATION: US 1999-421112 A1 19991019 (9) <--
PRIORITY: US 1998-104950P 19981020 (60)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 5 OF 22 USPATFULL on STN
2003:13075 Vaccine comprising an iscom consisting of sterol and saponin which is free of additional detergent.
Friede, Martin, Farnham, UNITED KINGDOM
Garcon, Nathalie, Wavre, BELGIUM
SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S. corporation)
US 6506386 B1 20030114
WO 2000007621 20000217
APPLICATION: US 2001-744800 20010604 (9) <--
WO 1999-EP5587 19990803 <--
PRIORITY: GB 1998-17052 19980805
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 6 OF 22 USPATFULL on STN
2002:332725 Compositions containing lysophosphotidic acids which inhibit apoptosis and uses thereof.
Bathurst, Ian C., Kensington, CA, United States
Foehr, Matthew W., San Francisco, CA, United States
Goddard, J. Graham, San Francisco, CA, United States
Umansky, Samiul R., Richmond, CA, United States
Bradley, John D., Brookoline, MA, United States
Picker, Donald H., Warren, NJ, United States
Sky High, LLC, Evanston, IL, United States (U.S. corporation)
US 6495532 B1 20021217
WO 9841213 19980924
APPLICATION: US 2000-381489 20000228 (9) <--
WO 1998-US5325 19980318 <--
PRIORITY: US 1997-39379P 19970319 (60)
US 1997-39380P 19970319 (60)
US 1997-39376P 19970319 (60)
US 1997-56120P 19970820 (60)
US 1997-56744P 19970820 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 7 OF 22 USPATFULL on STN
2002:202066 Compositions and methods for treating papillomavirus-infected cells
.
Howley, Peter M., Wellesley, MA, United States
Dowhanick-Morrissette, Jennifer J., Pottstown, PA, United States
Benson, John D., Brookline, MA, United States
Sakai, Hiroyuki, Kyoto, JAPAN
President and Fellows of Harvard College, Cambridge, MA, United States (U.S. corporation)
US 6432926 B1 20020813
APPLICATION: US 1999-362012 19990727 (9) <--
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 8 OF 22 USPATFULL on STN
2002:129535 Compositions and methods for treating Papillomavirus-infected cells
.
Howley, Peter M., Wellesley, MA, United States
Benson, John, Brookline, MA, United States
Kasukawa, Hiroaki, Princeton, NJ, United States
President and Fellows of Harvard College, Cambridge, MA, United States (U.S. corporation)
US 6399075 B1 20020604
APPLICATION: US 1999-347504 19990702 (9) <--
PRIORITY: US 1998-91661P 19980702 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 9 OF 22 USPATFULL on STN
2001:59871 Antisense oligonucleotide compositions and methods for the modulation of JNK proteins.
McKay, Robert, La Mesa, CA, United States
Dean, Nicholas, Olivenhain, CA, United States
Monia, Brett P., La Costa, CA, United States
Nero, Pamela Scott, Oceanside, CA, United States
Gaarde, William A., Carlsbad, CA, United States
Isis Pharmaceuticals Inc., Carlsbad, CA, United States (U.S. corporation)
US 6221850 B1 20010424
APPLICATION: US 1998-130616 19980807 (9) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 10 OF 22 USPATFULL on STN

2001:[illegible] Method for inducing a systemic immune response to an HIV antigen.
    See, Jackie R., Reno, NV, United States
    See, Darryl M., Laguna Niguel, CA, United States
    Bio-Sphere Technology, Reno, NV, United States (U.S. corporation)
    US 6207185 B1 20010327
    APPLICATION: US 1997-948568 19971010 (8) <--
    DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 11 OF 22 USPATFULL on STN
2000:138332 Antisense oligonucleotide compositions and methods for the
    modulation of JNK proteins.
    McKay, Robert, San Diego, CA, United States
    Dean, Nicholas, Olivenhain, CA, United States
    Monia, Brett P., La Costa, CA, United States
    Nero, Pamela S., Oceanside, CA, United States
    Gaarde, William A., Carlsbad, CA, United States
    Isis Pharmaceuticals Inc., Carlsbad, CA, United States (U.S. corporation)
    US 6133246 20001017
    APPLICATION: US 1999-287796 19990407 (9) <--
    DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 12 OF 22 USPATFULL on STN
2000:127960 Optoacoustic contrast agents and methods for their use.
    Unger, Evan C., Tucson, AZ, United States
    Wu, Yunqiu, Tucson, AZ, United States
    Imarx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
    US 6123923 20000926
    APPLICATION: US 1997-993165 19971218 (8) <--
    PRIORITY: US 1997-46379P 19970513 (60)
    DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 13 OF 22 USPATFULL on STN
2000:114120 Enhanced antisense modulation of ICAM-1.
    Bennett, C. Frank, Carlsbad, CA, United States
    Condon, Thomas P., Carlsbad, CA, United States
    Flournoy, Shin Cheng, San Diego, CA, United States
    Isis Pharmaceuticals Inc., Carlsbad, CA, United States (U.S. corporation)
    US 6111094 20000829
    APPLICATION: US 1998-62416 19980417 (9) <--
    DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 14 OF 22 USPATFULL on STN
2000:113925 DNA vaccines for eliciting a **mucosal** immune response.
    Malone, Robert W., Baltimore, MD, United States
    Malone, Jill G., Baltimore, MD, United States
    University of Maryland, Baltimore, Baltimore, MD, United States (U.S.
    corporation)
    US 6110898 20000829
    APPLICATION: US 1997-862632 19970523 (8) <--
    PRIORITY: US 1996-18269P 19960524 (60)
    DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 15 OF 22 USPATFULL on STN
2000:43772 Immunogenic composites capable of stimulating production of
    anti-peptide antibodies, pharmaceutical compositions employing these
    composites and methods of selectively inducing production of anti-peptide
    antibodies.
    Mannino, Raphael James, Newtonville, NY, United States
    Goodman-Snitkoff, Gail, Schenectady, NY, United States
    Albany Medical College, Albany, NY, United States (U.S. corporation)
    US 6048531 20000411

APPLICATION: US 1998-[illegible] (9)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 16 OF 22 USPATFULL on STN
1999:163506 Antisense modulation of LFA-3.
Bennett, C. Frank, Carlsbad, CA, United States
Condon, Thomas P., Carlsbad, CA, United States
Flournoy, Shin Cheng, San Diego, CA, United States
Pober, Jordan S., Guilford, CT, United States
Ma, Weillie, Hamden, CT, United States
Isis Pharmaceuticals Inc., Carlsbad, CA, United States (U.S. corporation)
US 6001651 19991214
APPLICATION: US 1998-45106 19980320 (9) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 17 OF 22 USPATFULL on STN
1999:113730 Antisense modulation of PECAM-1.
Bennett, C. Frank, Carlsbad, CA, United States
Condon, Thomas P., Carlsbad, CA, United States
Flournoy, Shin Cheng, San Diego, CA, United States
Zhang, Hong, Carlsbad, CA, United States
Isis Pharmaceuticals Inc., Carlsbad, CA, United States (U.S. corporation)
US 5955443 19990921
APPLICATION: US 1998-44506 19980319 (9) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 18 OF 22 USPATFULL on STN
1999:27762 Antisense oligonucleotides against JNK.
McKay, Robert, La Mesa, CA, United States
Dean, Nicholas M., Encinitas, CA, United States
ISIS Pharmaceuticals, Inc., Carlsbad, CA, United States (U.S. corporation)
US 5877309 19990302
APPLICATION: US 1997-910629 19970813 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 19 OF 22 USPATFULL on STN
1998:108397 Methods and devices for immunizing a host through administration of naked polynucleotides with encode allergenic peptides.
Carson, Dennis A., Del Mar, CA, United States
Raz, Eyal, San Diego, CA, United States
Howell, Meredith L., Corvallis, OR, United States
The Regents of the University of California, Alameda, CA, United States (U.S. corporation)
US 5804566 19980908
APPLICATION: US 1994-333068 19941101 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 20 OF 22 USPATFULL on STN
1998:14497 Solid fat nanoemulsions as vaccine delivery vehicles.
Anselem, Shimon, Rehovot, Israel
Lowell, George H., Baltimore, MD, United States
Aviv, Haim, Rehovot, Israel
Friedman, Doron, Carmei Yosef, Israel
Pharmos Corporation, New York, NY, United States (U.S. corporation)The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 5716637 19980210
WO 9426255 19941124
APPLICATION: US 1995-553350 19951116 (8) <--
WO 1994-US5589 19940518 19951116 PCT 371 date 19951116 PCT 102(e) date<--
DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 21 OF 22 USPATFULL on STN
95:92774 Cationic lipids for intracellular delivery of biologically active molecules.
Felgner, Philip L., Rancho Santa Fe, CA, United States
Kumar, Raj, San Diego, CA, United States
Basava, Channa, San Diego, CA, United States
Border, Richard C., Poway, CA, United States
Hwang-Felgner, Jiin-Yu, Rancho Santa Fe, CA, United States
Vical, Inc., San Diego, CA, United States (U.S. corporation)
US 5459127 19951017
APPLICATION: US 1993-123757 19930916 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L9 ANSWER 22 OF 22 USPATFULL on STN
91:75536 Novel **liposome** composition for the treatment of interstitial lung diseases.
Radhakrishnan, Ramachandran, Fremont, CA, United States
Liposome Technology, Inc., Menlo Park, CA, United States (U.S. corporation)
US 5049389 19910917
APPLICATION: US 1989-444738 19891201 (7) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d l9,cbib,ab,clm,kwic,21,15,10

L9 ANSWER 21 OF 22 USPATFULL on STN
95:92774 Cationic lipids for intracellular delivery of biologically active molecules.
Felgner, Philip L., Rancho Santa Fe, CA, United States
Kumar, Raj, San Diego, CA, United States
Basava, Channa, San Diego, CA, United States
Border, Richard C., Poway, CA, United States
Hwang-Felgner, Jiin-Yu, Rancho Santa Fe, CA, United States
Vical, Inc., San Diego, CA, United States (U.S. corporation)
US 5459127 19951017
APPLICATION: US 1993-123757 19930916 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Disclosed are cationic lipids capable of facilitating transport of biologically active agents into cells, including the transfection of cells by therapeutic polynucleotides, the delivery of antiviral drugs, and the introduction of immunogenic peptides. The cationic lipids, comprising an ammonium group, have the general structure ##STR1## Also disclosed are adducts of these compounds comprising additional cationic sites that enhance the transfective or transport activity. Structure-activity correlations provide for the selection of preferred compounds to be synthesized for this purpose. Compositions disclosed for use of these cationic lipid include formulations for in vitro transfection and pharmaceutical formulations for parenteral and topical administration of therapeutic agents.

CLM What is claimed is:

1. A formulation for transfection of polynucleotides into cells, comprising a cationic lipid and an effective transfection-promoting amount of a lysophosphatide, having the structure ##STR20## wherein Y is selected from the group consisting of --O--CH2-- and --O--C(O)--; R is C_{10} to C_{23} alkyl or alkenyl; and Z is a phospholipid headgroup.

2. A formulation for transfection of polynucleotides and peptides into cells, comprising a compound having the structure ##STR21## wherein Y^1 and Y^2 are the same or different and are --O--C(O)-- or --O--; R^1 is H, or C_1 to C_{24} alkyl or alkenyl; R^2 is

C_1 to C_{24} alkyl or alkenyl; R^3 and R^4 are independently C_1 to C_{24} alkyl or H; R^5 is C_1 to C_{24} alkyl straight chain or branched chain; R^6 is --C(O)--$(CH_2)_m$ --NH--, a diaminocarboxylate ester group which is alkyl, aryl, or aralkyl, or --C(O)--$(CH_2)_m$ --NH-- linked to said diaminocarboxylate ester group, or is absent; R^7 is H, spermine, spermidine, a histone, or a protein with DNA-binding specificity, or the same groups wherein the amine functionalities of the R^7 moiety are quaternized with R^3, R^4, or R^5 groups; or R^7 is an L- or D-alpha amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or analogues thereof, or wherein the amine of the R^7 moiety is quaternized with R^3, R^4 or R^5 groups; or R^7 is a polypeptide selected from the group consisting of L- or D-alpha amino acids, wherein at least one of the amino acids residues comprises arginine, histidine, lysine, ornithine, or analogues thereof; n is 1 to 8; m is 1 to 18; and X is non-toxic anion.

3. A formulation for transfection of polynucleotides and peptides into cells, comprising a compound having the structure ##STR22## or an optical isomer thereof, wherein Y^1 and Y^2 are different and are either --O--C(O)-- or --O--; R^1 is C_1 to C_{24} alkyl or alkenyl, or H; R^2 is C_1 to C_{24} alkyl or alkenyl; R^3, R^4 and R^5 are independently H, C_1 to C_{14} alkyl, C_7 to C_{11} aryl or alkaryl, or at least two of R^3, R^4 and R^5 are taken together to form quinuclidino, piperidino, pyrrolidino, or morpholino; and X is a non-toxic anion; together with an effective transfection-promoting amount of a lysophosphatide.

4. A formulation for transfection of polynucleotides and peptides into cells, comprising a compound selected from the group consisting of 3,5-(N,N-dilysyl)-diaminobenxzoyl-3-(DL-1,2-dioleoyldimethylaminopropyl-β-hydroxyethylamine; 3,5-(N,N-dilysyl)diaminobenxzoylglycyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl-β-hydroxyethylamine); and L-spermine-5-carboxyl-3-(DL-1,2-dioleoyldimethylaminopropyl-β-hydroxyethylamine) together with an effective transfection-promoting amount of a lysophosphatide.

5. A formulation according to claims 2 or 3, wherein said lysophosphatide is selected from the group consisting of lysophosphatidylcholine and lysophosphatidylethanolamine.

6. A formulation according to claim 5, wherein said lysophosphatide is a mono-oleoyl lysophosphatidylcholine.

7. A formulation according to claim 2 or 3, wherein said lysophosphatide has a negatively charged headgroup.

8. A formulation according to claim 2 or 3, wherein said lysophosphatide has a neutrally charged headgroup.

9. A formulation according to claim 2 or 3, wherein the molar ratio of said lysophosphatide to cationic lipid is less than about 0.50.

10. A formulation comprising a cationic lipid and a lysophosphatide, wherein said cationic lipid is selected from the group consisting of DOTMA, DOTAP, and compounds having a structure set forth in claim 2 or 3.

11. A lipid formulation for the transfection of polynucleotides and peptides into cells, comprising a cationic lipid or combination of cationic lipids selected from the group consisting of DOTMA, DOTAP, or a

structure according to claim 2 or 3, wherein an effective transfection-promoting amount of said cationic lipids up to about one-third of the total cationic lipids are selected from species wherein Y^1 and R^1 or Y^2 and R^2 of said structure, or moieties of DOTMA or DOTAP corresponding thereto, are hydroxyl groups.

12. A liposomal formulation, comprising a cationic lipid according to claim 2 or 3, or a mixture of said cationic lipids, said lipid or mixture of lipids being in the form of vesicles in an aqueous media.

13. A liposomal formulation according to claim 12, further comprising a neutral lipid species, selected from the group consisting of phosphatidylethanolamine, **phosphatidylcholine**, sphingomyelin, or **cholesterol**.

14. A liposomal formulation according to claim 13 wherein the molar ratio of said cationic lipid species to said neutral lipid species is from about 9/1 to 1/9.

15. A liposomal formulation according to claim 13 wherein said molar ratio is about 5/5.

16. A liposomal formulation according to claim 13 further comprising a lyso lipid selected from the group consisting of lysophosphatidylcholine, lysophosphatidylethanolamine, or a lyso form of a cationic lipid species.

17. A pharmaceutical product comprising a cationic lipid having a structure set forth in claim 2 or 3, together with a pharmacologically effective amount of therapeutic agent.

18. A pharmaceutical product according to claim 17, wherein said therapeutic agent is a corticosteroid or a non-steroidal anti-inflammatory agent.

19. A pharmaceutical product according to claim 17, wherein said therapeutic agent is a therapeutically effective nucleoside analogue or nucleotide analogue.

20. A pharmaceutical product according to claim 18, wherein said therapeutic agent is a phosphatidyl derivative or diphosphate diglyceride derivative of said analogue.

21. A pharmaceutical product according to claim 17, wherein said analogue is dideoxynucleoside, a didehydronucleoside, a halogenated or azido derivative of a nucleoside, or an acyclic nucleoside.

22. A pharmaceutical product according to claim 19, wherein said analogue is an antiviral nucleoside selected from the group consisting of a 3'-azido-2',3'-dideoxypyrimidine, a 3'-halopyrimidine dideoxynucleoside, or a 2',3'-didehydro-2',3'-dideoxynucleoside.

23. A pharmaceutical product according to claim 22, wherein said analogue is an antiviral nucleoside which is 3'-azido-3'-deoxythymidine (AZT).

24. A pharmaceutical product according to claim 21, wherein said analogue is an antiviral nucleoside selected from the group consisting of acyclovir, gancyclovir, 1-(2-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) or 1(2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)5-iodouracil (FIAU).

25. A pharmaceutical product according to claim 19, comprising a therapeutic polynucleotide.

26. A pharmaceutical product according to claim 25, wherein said

therapeutic polynucleotide is a ribozyme or an antisense RNA or DNA.

27. A pharmaceutical preparation according to claim 26, wherein said ribozyme or antisense DNA or RNA is directed against **HIV**.

28. A pharmaceutical preparation according to claim 26, wherein said ribozyme or antisense DNA or RNA is directed against the rev transactivator.

29. A pharmaceutical preparation according to claim 25, wherein said therapeutic polynucleotide is a 28-mer phosphorothioate antisense polynucleotide.

30. A pharmaceutical preparation according to claim 25, wherein said therapeutic polynucleotide codes for a therapeutic polypeptide.

31. The pharmaceutical preparation of claim 30, wherein said therapeutic polypeptide is deficient or absent in a disease state.

32. The pharmaceutical preparation of claim 30, wherein said therapeutic polypeptide is a natural hormone or a synthetic analog thereof.

33. The pharmaceutical preparation of claim 30, wherein said therapeutic polypeptide is an immunogen.

34. The pharmaceutical preparation of claim 27, wherein said therapeutic agent is a protein or peptide.

35. A pharmaceutical preparation for topical use comprising a compound having the structure set forth in claim 2 or 3, and a pharmacologically effective amount of a therapeutic agent in a pharmaceutically acceptable vehicle.

36. The pharmaceutical preparation of claim 25, wherein said therapeutic agent is a corticosteroid, a nonsteroidal anti-inflammatory agent, an antibiotic, an antifungal agent, an oxidant, or an antiviral nucleoside.

37. The pharmaceutical preparation of claim 25, wherein said therapeutic agent is a protein, a polypeptide, or a therapeutic polynucleotide.

38. The pharmaceutical preparation of claim 25 comprising a therapeutic polynucleotide which is a ribozyme or an antisense RNA or DNA sequence.

39. The pharmaceutical preparation of claim 25 comprising a therapeutic polynucleotide coding for a gene product that is deficient or absent in a disease state.

40. The pharmaceutical preparation of claim 25 comprising a therapeutic polynucleotide coding for an immunogenic peptide, a natural hormone, or a synthetic analogue of a natural hormone.

41. A pharmaceutical preparation for topical use in the treatment of Herpes simplex, comprising a compound having the structure set forth in claim 2 or 3 together with a pharmacologically effective concentration of acyclovir, gancyclovir, 1-(2-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) or 1(2'-deoxy-2'-fluoro-1-β-D-arabinofuranosyl)5-iodouracil (FIAU) in a pharmaceutically acceptable vehicle.

42. A method for introducing a biologically active agent into a cell of a plant or animal, comprising: a. preparing lipid vesicles comprising a cationic lipid having a structure set forth in claim 2 or 3 and containing said biologically active agent; b. contacting said cell with said lipid vesicles whereby said biologically active agent is taken up into said cell.

43. A method for introducing a biologically active agent into a cell of a plant or animal, comprising: preparing lipid vesicles comprising a cationic lipid having a structure set forth in claim 2 or 3; contacting said cell with a bioactive agent in the presence of said lipid vesicles, whereby said bioactive agent is taken up into said cell.

44. A method according to claim 43, wherein said contacting step occurs in vitro.

45. A method of treating a disease in a vertebrate, comprising the step of administering a pharmaceutical preparation comprising a cationic lipid having a structure set forth in claim 2 or 3 and a pharmacologically effective amount of a therapeutic agent specific for the treatment of said disease, and permitting said therapeutic agent to be incorporated into at least one cell of said vertebrate, whereby said disease is effectively treated.

46. A method according to claim 45, comprising the administration of said preparation to the cells of said vertebrate in vitro, which cells are then returned to said vertebrate.

47. A method according to claim 45, comprising the topical application of said preparation to the skin or to a **mucosal** surface.

48. A method according to claim 45, comprising the injection of said preparation into body cavity or into the tissues of said vertebrate.

49. A method according to claim 45, comprising the **oral** administration of said preparation.

50. A method according to claim 45, wherein said biologically active agent is a polynucleotide.

51. A method according to claim 45, wherein said biologically active agent is DNA or mRNA coding for a polypeptide, and said polypeptide is expressed after said DNA or said mRNA is taken up into said cell.

52. A method according to claim 45 wherein said biologically active agent is a drug.

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SUMM . . . passage of potentially beneficial therapeutic substances into the body. The complex composition of the cell membrane comprises phospholipids, glycolipids, and **cholesterol**, as well as intrinsic and extrinsic proteins, and its functions are influenced by cytoplasmic components which include Ca++ and other. . .

SUMM . . . of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form hollow lipid vesicles, or **liposomes**, in aqueous systems. This property can be used to entrap the substance to be delivered within the **liposomes**; in other applications, the drug molecule of interest can be incorporated into the lipid vesicle as an intrinsic membrane component,. . .

SUMM **Liposomes** have been discussed as possible in vivo delivery vehicles and some encouraging results using this approach to the intracellular expression. . . Acad. Sci. USA 84 7851-7855 (1987)); however, the methodology has fundamental problems. Chief among the difficulties is the failure of **liposomes** to fuse with the target cell surface, but to be taken up phagocytically instead. Phagocytized **liposomes** are delivered to the lysosomal compartment, where polynucleotides are subjected to the action of digestive enzymes and degraded, leading to. . .

SUMM . . . in this area was the discovery that a positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of **liposomes**, or small vesicles, could interact spontaneously with DNA to form lipid-DNA complexes which are capable of fusing with the negatively. . . Md.),

an effective agent for the delivery of highly anionic polynucleotides into living tissue culture cells comprises positively charged DOTMA **liposomes** which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged **liposomes** are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously. . .

SUMM Although the use of known cationic lipids overcomes many problems associated with conventional **liposome** technology for polynucleotide delivery in vitro, several problems related to both in vitro and in vivo applications remain. First, although. . .

SUMM . . . that a cationic lipid (DOTAP), originally synthesized by Eibl (Eibl, H. and Woolley, P. Biophys. Chem. 10 261-271 (1979)) forms **liposomes** which can fuse with negatively charged **liposomes** and can deliver functional DNA and RNA into tissue culture fibroblasts (Stamatatos, L., Leventis, R., Zuckermann, M. J. & Silvius,. . .

DRWD FIGS. 9a-9c demonstrate the effect of **cholesterol** in the transfection lipid formulation on the efficiency of DNA transfection.

DETD . . . media. The lipids of the liposomal formulation can further comprise a neutral lipid species selected from the group consisting of **phosphatidylcholine**, phosphatidylethanolamine, sphingomyelin, or **cholesterol**. A preferred molar ratio of cationic to neutral lipid species in these formulations is from about 9/1 to 1/9; a. . .

DETD . . . antisense RNA or DNA. In preferred embodiments, the formulation comprises an antisense DNA or RNA or a ribozyme directed against **HIV**. In a particularly preferred embodiment, the therapeutic polynucleotide is an antisense DNA or RNA or a ribozyme directed against the rev transactivator of **HIV**. An example of such an agent is the 28-mer phosphorothioate antisense polynucleotide. Alternatively, the therapeutic polynucleotide can be one coding. . .

DETD . . . or encapsulating the bioactive agent in the lipid vesicle and contacting the cell with the lipid vesicles, as in conventional **liposome** methodology; or alternatively, by contacting the cells simultaneously with empty lipid vesicles, comprising the cationic lipids together with the bioactive. . .

DETD . . . the skin; the injection of a preparation into a body cavity or into the tissues of said vertebrate; or the **oral** administration of said preparation. The biologically active agent can be a polynucleotide, such as, for example, DNA or mRNA coding. . .

DETD . . . alkyl groups, as well as adducts of these cationic lipids, are advantageously used in formulations to prepare lipid vesicles or **liposomes** to be used in transfection procedures, or to similarly facilitate the intracellular delivery of proteins, polypeptides, small organic molecules, and. . .

DETD . . . aspect of the invention, the CLs are combined with other lipids in formulations for the preparation of lipid vesicles or **liposomes** for use in intracellular delivery systems. The formulations preferably are prepared from a mixture of positively charged lipids, negatively charged lipids, neutral lipids and **cholesterol** or a similar sterol. The positively charged lipid can be one of the cationic lipids of the invention alone, a. . . synthetic phospholipids or mono-, di-, or triacylglycerols. The natural phospholipids are typically those from animal and plant sources, such as **phosphatidylcholine**, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, or phosphatidylinositol. Synthetic phospholipids typically are those having identical fatty acid groups, including, but not limited to, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine and the corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. The neutral lipid can be **phosphatidylcholine**, cardiolipin, phosphatidylethanolamine, mono-, di- or triacylglycerols, or analogues thereof. The negatively charged lipid can be phosphatidylglycerol, phosphatidic acid or a similar phospholipid analog. Other additives such as **cholesterol**, glycolipids, fatty acids, sphingolipids, prostaglandins, gangliosides, neobee, niosomes, or any other natural or synthetic amphophiles can also be used in **liposome**

formulations, as is conventionally known for the preparation of **liposomes**.

DETD . . . 0 to 95 mole %, and most preferably 0 to 80 mole %. In order to produce lipid vesicles or **liposomes** having a net positive charge, the quantity of the positively charged component must exceed that of the negatively charged component.. . . charged lipid can be present at between about 0 to 49 mole % and preferably 0 to 40 mole %. **Cholesterol** or a similar sterol can be present at 0 to 80 mole %, and preferably 0 to 50 mole %.

DETD Lipid formulations comprising at least one amphipathic lipid can spontaneously assemble to form primary **liposomes**, heterogeneous in size. Therefore, according to a preferred method, the lipid reagents of the invention, comprising at least one cationic lipid species, are prepared as **liposomes** according to the procedure of Example 12. The component lipids are dissolved in a solvent such as chloroform and the. . . inner surface of a glass vessel. On suspension in an aqueous solvent, the amphipathic lipid molecules assemble themselves into primary **liposomes**. If other molecules are present in the aqueous solvent, such as, for example, a bioactive substance, these will be captured within the **liposomes**. Otherwise, empty **liposomes** will be formed.

DETD A bioactive substance in the form of its lipid derivative, may be added to the component lipids of the **liposome** formulation, to be incorporated into the wall of the **liposomes** on hydration.

DETD These primary **liposomes** are reduced to a selected mean diameter by means of the freeze-thaw procedure referred to above. The CLs of the. . . vesicle production published in the literature and known to those in the art, for example, the sonication of spontaneously formed **liposomes** comprised of the lipids in aqueous solution described by Felgner, P. L. et al., Proc. Natl. Acad. Sci., USA 84:7413-7417. . . Wilschut et al. Biochemistry 19:6011-6021(1980) or freeze-thaw and extrusion (Mayer, L. et al., Biochim. Biophys. Acta 858:161-168 (1986). To prepare **liposomes** suitable for physiological in vivo use, having a **unilamellar** structure and a uniform size of from about 50 to about 200 μm in diameter, the primary **liposomes** are preferably processed by the freeze-thaw and extrusion processes.

DETD . . . reduce the efficiency of transfection. The presence of DOPE or DOPC reduced the effectiveness of DOTMA in RNA transfection, while **cholesterol** was less inhibitory (Examples 17 and 18; FIGS. 3-4). However, DORI was most effective in DNA transfection when combined with. . .

DETD . . . combination with other known cationic lipids such as for example, DOTMA or DOTAP, in any procedure comprising the use of **liposomes** or lipid vesicles to deliver substances intracellularly either in vitro or in vivo. Those lipids having metabolizable ester bonds are. . .

DETD . . . 570:220-241 (1987). Many of these oligonucleotide species, which are scarce and expensive to synthesize, are inefficiently captured by encapsulation into **liposomes** of negatively charged lipids, according to ordinary current methods. We have experimental studies showing that these oligonucleotides are captured within cationic **liposomes** with efficiencies approaching 100%. Also within the scope of the invention is the delivery, by means of the cationic lipids. . .

DETD . . . of either an antisense polynucleotide or ribozyme as described above, and having as its target the rev site of the **HIV** genome (Scientific American, October, 1988, pp. 56-57). Matsukura, M. et al. Proc. Nat'l. Acad. Sci. 86:4244-4248 (1989) describe a 28-mer phosphorothioate compound anti-**HIV** (anti-rev transactivator) specific for the site.

DETD . . . analogues as diphosphate diglyceride derivatives. Preferred species of lipid derivatives of antiviral or antiretroviral nucleoside analogues for the treatment of **HIV** infection using cationic lipid mediated liposomal delivery are phospholipid derivatives of 3'-azido-2',3'-dideoxypyrimidine, 3'-halopyrimidine dideoxynucleoside, or a 2',3'-didehydro-2',3'-dideoxynucleoside, for example, phosphatidyl. . .

DETD . . . agents, particularly the phosphatidylglycerol derivatives as disclosed in U.S. application Ser. No. 373,088, and as such may be incorporated into **liposomes** comprising one or more cationic lipids of the invention.

DETD . . . Biophys. Acta 917:33-41 (1987). This feature of the ester/ether species of cationic lipids is significant in view of studies indicating **liposome** mediated transfection can occur with significant efficiency in vivo, for example in infusion into the trachea (Brigham, K. L. et. . .

DETD . . . incorporated herein by reference. Cationic lipid reagents of the invention may be prepared and stored in aqueous solution as empty **liposomes**, or may be stored dry after formulation to be later used as encapsulating agents for selected bioactive substances.

DETD . . . procedures of Examples 7, 8 and 9. The cationic lipid DOTMA was combined in formulations either alone or combined with **cholesterol** and compared with similar formulations comprising the neutral lipid DOPE as indicated in the table of Example 18 below. The highest activity occurs in formulations lacking the phospholipid component, particularly in the presence of **cholesterol**. FIG. 4 taken from the same set of transfections, indicates that the newly defined cationic lipid composition (DOTMA/DOPE/**Cholesterol** 70/0/30), containing no phospholipid, gives rise to much higher levels of mRNA expression (compare the scales on the y-axis of. . .

DETD . . . of cationic lipids was evaluated under optimal transfection conditions determined as described; that is using lipid formulations in the ratio CL/**cholesterol** of 70/30 with no phospholipid component, and allowing the first stage association of lipid vesicles and mRNA to occur in. . .

DETD PREPARATION OF **LIPOSOME**-FORMING DOTAP

DETD The cationic **liposome**-forming material 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) may be also prepared as reported by L. Stamatatos, et al. Biochemistry 27:3917-3925 (1988) or H. Eibl. . .

DETD . . . charged vesicles can be prepared without sonication to produce multilamellar vesicles (MLV) or by extrusion through nuclepore membranes to produce **unilamellar** vesicles of a discrete size. Other methods are also available and are known to those familiar with the art. DOTMA. . .

DETD . . . were prepared by mixing 0.5 ml of a 10 ug/ml polynucleotide solution with 0.5 ml of sonicated DOTMA/PE or DOTAP/PE **liposomes** at 40-100 ug/ml by slow addition through a syringe with constant gentle vortexing. The diluted polynucleotide and **liposome** solutions are prepared from concentrated stock solutions by dilutions into Opti-MEM Reduced Serum Media obtained from Gibco/BRL, Gaithersburg Md., at. . . results in positively charged complexes which, will spontaneously deliver polynucleotide into cells in tissue culture. Different ratios of positively charged **liposomes** to polynucleotides can be used to suit the need. These methods are essentially as described in Felgner, P. L. et. . .

DETD . . . either dioleoyl phosphatidyl ethanolamine (DOPE) or dioleoyl phosphatidyl choline (DOPC). Each formulation was prepared with and without 33 mole % **cholesterol**. Four different levels of lipid were tested: 50, 75, 100, and 125 μg; at a fixed RNA level of 20. . .

DETD

		Luciferase light units Mole percent CL:CL + Phospholipid 20%	50%	80%
DOPE	DOTMA	86	830	4268
	DOTAP	1	536	1066
	DOTMA:**Cholesterol** 7:3	127	1102	1568
	DOTAP:**Cholesterol** 7:3	2	77	784
DOPC	DOTMA	2	51	69
	DOTAP	2	3	51

DOTAP:**Cholesterol** 7:3
48 522 501
0 88 256

DETD (4) **Cholesterol** does not have a dramatic inhibitory effect in these formulations.

DETD . . . data indicates that formulations consisting of either 100 mole % DOTMA or 70 mole % DOTMA and 30 mole % **cholesterol** give rise to the highest activity in the absence of serum. Data below indicate that the best activity in the presence of serum occurs with the formulations containing **cholesterol**. For example, the replacement of 30% of the DOTMA in 100/0/0 by **cholesterol** in the formulation 70/0/30 demonstrates marked enhancement of activity due to the presence of **cholesterol**.

DETD . . . Lipofectin.TM.. However, in this experiment, all lipid formulations were prepared with 70 mole % cationic lipid and 30 mole % **cholesterol**, a ratio which is shown to make the DOTMA formulation used herein 3-4 fold more active than the Lipofectin.TM. reagent. . .

DETD B: **Cholesterol**

DETD **Cholesterol** (CHOL) was added to DORI at a molar ratio of DORI/CHOL 7/3 and the lipid formulation used to transfect COS.7. . .

. . . A liposomal formulation according to claim 12, further comprising a neutral lipid species, selected from the group consisting of phosphatidylethanolamine, **phosphatidylcholine**, sphingomyelin, or **cholesterol**.

27. A pharmaceutical preparation according to claim 26, wherein said ribozyme or antisense DNA or RNA is directed against **HIV**.

47. A method according to claim 45, comprising the topical application of said preparation to the skin or to a **mucosal** surface.

49. A method according to claim 45, comprising the **oral** administration of said preparation.

L9 ANSWER 15 OF 22 USPATFULL on STN

2000:43772 Immunogenic composites capable of stimulating production of anti-peptide antibodies, pharmaceutical compositions employing these composites and methods of selectively inducing production of anti-peptide antibodies.

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APPLICATION: US 1993-160093 19931201 (8) <--
DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB An essentially pure immunogenic composite capable of selectively inducing antibody production in an animal to a peptide that if administered by itself to the animal without being conjugated to or mixed with additional substance does not stimulate production of anti-peptide antibody in the animal. This immunogenic composite comprises: a lipid and an amphipathic peptide. The amphipathic peptide if administered by itself to the animal without being conjugated to or mixed with additional substance does not stimulate production of anti-peptide antibody in the animal. The amphipathic peptide is covalently bound to the lipid in a peptide-lipid complex. The immunogenic composite has a vesicular or an amorphous particulate structure. Another embodiment of this immunogenic composite comprises: a lipid, the amphipathic peptide, and a hydrophilic peptide covalently bound together. The hydrophilic peptide if administered by itself to the animal without being conjugated to or mixed with additional substance does not stimulate production of anti-peptide antibody in the animal.

CLM What is claimed is:

1. An essentially pure immunogenic composition comprising: (A) a lipid, which is one member selected from the group consisting of a phospholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalently bound to (B) an amphipathic peptide that primes homologous helper T-cells and that when administered by itself to a animal does not stimulate production of anti-peptide antibody in said animal; wherein, when said lipid is a phospholipid or sphingolipid, said amphipathic peptide is covalently bound to a head group of said phospholipid or sphingolipid via a cross-linker, and wherein said immunogenic composition selectively induces antibody production in said animal to said amphipathic peptide.

2. The essentially pure immunogenic composition of claim 1, further comprising at least two additional lipids, at least one of which is a sterol.

3. An essentially pure immunogenic composition comprising: (A) a lipid, which is one member selected from the group consisting of a phospholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalently bound to (B) a first hybrid peptide that is (1) an amphipathic peptide that primes homologous helper T-cells and that when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, covalently bound to (2) one peptide selected from the group of peptides consisting of: (a) a hydrophilic peptide, (b) a neutral peptide, (c) an amphipathic peptide, and (d) a second hybrid peptide comprising any combination of two or more of the above-defined peptides (a), (b) or (c), wherein each of said peptides (a), (b), (c) and (d) primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, wherein, when said lipid is a phospholipid or spingolipid, said first hybrid peptide is covalently bound to a head group of said phospholipid or sphingolipid via a cross-linker, and wherein said immunogenic composition selectively induces antibody production in said animal to said peptides comprising said first hybrid peptide.

4. The essentially pure immunogenic composition of claim 3, further comprising at least two additional lipids, at least one of which is a sterol.

5. The essentially pure immunogenic composition of claim 3, wherein said first hybrid peptide comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

6. The essentially pure immunogenic composition of claim 4, wherein said first hybrid peptide comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

7. The essentially pure immunogenic composition of claim 1, wherein said lipid (A) is phosphatidylethanolamine.

8. The essentially pure immunogenic composition of claim 2, wherein said lipid (A) is phosphatidylethanolamine.

9. The essentially pure immunogenic composition of claim 3, wherein said lipid (A) is phosphatidylethanolamine.

10. The essentially pure immunogenic composition of claim 4, wherein said lipid (A) is phosphatidylethanolamine.

11. The essentially pure immunogenic composition of claim 2, wherein said lipid (A) is **cholesterol**.

12. The essentially pure immunogenic composition of claim 4, wherein said lipid (A) is **cholesterol**.

13. An essentially pure immunogenic composition comprising at least two immunogenic compositions selected from the group consisting of: (I) an essentially pure immunogenic composition comprising: (A) a lipid, which is one member selected from the group consisting of a phospholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalently bound to (B) an amphipathic peptide that primes homologous helper T-cells and that when administered by itself to an animal not stimulate production of anti-peptide antibody in said animal; wherein, when said lipid (I)(A) is a phospholipid or sphingolipid, said amphipathic peptide is covalently bound to a head group of said phospholipid or sphingolipid via a cross-linker, and wherein said immunogenic composition (I) selectively induces antibody production in said animal to said amphipathic peptide; (II) said essentially pure immunogenic composition (I), further comprising at least two additional lipids, at least one of which is a sterol; (III) an essentially pure immunogenic composition comprising: (A) a lipid, which is one member selected from the group consisting of a phospholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalently bound to (B) a first hybrid peptide that is (1) an amphipathic peptide that primes homologous helper T-cells and that when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, covalently bound to (2) one peptide selected from the group of peptides consisting of: (a) a hydrophilic peptide, (b) a neutral peptide, (c) an amphipathic peptide, and (d) a second hybrid peptide comprising combination of two or more of the above-defined peptides (a),(b) or (c), wherein each of said peptides (a), (b), (c) and (d) primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of an anti-peptide antibody in said animal, wherein, when said lipid (III)(A) is a phospholipid or sphingolipid, said first hybrid peptide is covalently bound to head group of said phospholipid or sphingolipid via a cross-linker, and wherein said immunogenic composition (III) selectively induces antibody production in said animal to said peptides comprising said first hybrid peptide; and (IV) said essentially pure immunogenic composition (III), further comprising at least two additional lipids, at least one of which is a sterol.

14. An essentially pure immunogenic composition comprising at least two immunogenic compositions selected from the group consisting of: (I) an essentially pure immunogenic composition comprising: (A) a lipid, which is one member selected from the group consisting of a phosopholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalently bound to (B) an amphipathic peptide that primes homologous helper T-cells and that when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal; wherein, when said lipid (I)(A) is a phospholipid or sphingolipid, said amphipathic peptide is covalently bound to ahead group of said phospholipid or sphingolipid via a cross-linker, and wherein said immunogenic composition (I) selectively induces antibody production in said animal to said amphipathic peptide, (II) said essentially pure immunogenic composition (I), further comprising at least two additional lipids, at least one of which is a sterol; (III) an essentially pure immunogenic composition comprising; (A) a lipid, which is one member selected from the group consisting of a phospholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalently bound to (B) a first hybrid peptide that is (1) an amphipathic peptide that primes homologous helper T-cell and that when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, covalently bound to (2) one peptide selected from the group of peptides consisting of; (a) a hydrophilic peptide, (b) a neutral peptide, (c) an amphipathic peptide, and (d) a second hybrid peptide comprising any combination of two or more of the above-defined peptides (a), (b) or (c), wherein each of said peptides (a), (b), (c) and (d) primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of anti-peptide antibody in said

animal, wherein, when said lipid (III)(A) is a phospholipid or sphingolipid, said first hybrid peptide is covalently bound to a head group of said phospholipid or sphingolipid via a cross-linker, and wherein said immunogenic composition (III) selectively induces antibody production in said animal to said peptides comprising said first hybrid peptide; (IV) said essentially pure immunogenic composition (III), further comprising at least two additional lipids, at least one of which is a sterol; and (V) a composition comprising: (A) said immunogenic composition (I) or (II), and (B) a composition comprising: (1) a lipid, which is one member selected from the group consisting of a phospholipid, sphingolipid, and sterol, each of which is a component of membranes of eukaryotic or prokaryotic cells, covalenty bound to (2) at least one member selected from the group consisting of: (a) a hydrophilic peptide that primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, (b) a neutral peptide that primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, (c) an amphipathic peptide that primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, and (d) a third hybrid peptide that is (i) an amphipathic peptide that primes homologous helper T-cells and when administered by itself to animal does not stimulate production of anti-peptide antibody in said animal, covalently bound to (ii) one peptide selected from the group consisting of (aa) a hydrophilic peptide, (bb) a neutral peptide, (cc) an amphipathic peptide, and (dd) a fourth hybrid peptide comprising two or more of the said peptides (aa), (bb) or (cc), wherein each of said peptides (aa), (bb), (cc) and (dd) primes homologous helper T-cells and when administered by itself to an animal does not stimulate production of anti-peptide antibody in said animal, wherein, when said lipid (V)(B)(2)is a phospholipid or sphingolipid, said hydrophilic peptide (a), said neutral peptide (b), said amphipathic peptide (c) or said first hybrid peptide (d) is covalently to bound a head group of said phospholipid or sphingolipid via a cross-linker, and (3) one or more lipids and one or more sterols.

15. The essentially pure immunogenic composition of claim 14, wherein said lipid (A) in said composition (I), (II), (III), (IV), or (V)(A) is phosphatidylethanolamine.

16. The essentially pure immunogenic composition of claim 14, wherein said lipid (A) in said composition (I), (II), (III), (IV), or (V)(A)is **cholesterol**.

17. The essentially pure immunogenic composition of claim 14, wherein said first hybrid peptide in said immunogenic composition (III) or (IV) comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

18. The essentially pure immunogenic composition of claim 1, wherein said lipid (A) is a phospholipid.

19. The essentially pure immunogenic composition of claim 1, wherein said lipid (A) is a sphingolipid.

20. The essentially pure immunogenic composition of claim 1, wherein said lipid (A) is a sterol.

21. The essentially pure immunogenic composition of claim 3, wherein said lipid (A) is a phospholipid.

22. The essentially pure immunogenic composition of claim 3, wherein said lipid (A) is a sphingolipid.

23. The essentially pure immunogenic composition of claim 3, wherein said lipid (A) is a sterol.

24. The essentially pure immunogenic composition of claim 14, wherein said lipid (A) is a phospholipid.

25. The essentially pure immunogenic composition of claim 14, wherein said lipid (A) is a sphingolipid.

26. The essentially pure immunogenic composition of claim 14, wherein said lipid (A) is a sterol.

27. A pharmaceutical composition for selectively inducing antibody production, said composition comprising: (A) the immunogenic composition of claim 1, and (B) a pharmaceutically acceptable carrier, diluent or excipient.

28. A pharmaceutical composition for selectively inducing antibody production, said composition comprising: (A) the immunogenic composition of claim 3, and (B) a pharmaceutically acceptable carrier, diluent or excipient.

29. The pharmaceutical composition of claim 28, wherein said first hybrid peptide comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

30. A pharmaceutical composition for selectively inducing antibody production, said composition comprising: (A) the immunogenic composition of claim 14, and (B) a pharmaceutically acceptable carrier, diluent or excipient.

31. The pharmaceutical composition of claim 30, wherein said first hybrid peptide in said immunogenic composition (III) or (IV) comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

32. The pharmaceutical composition of claim 27, 28, or 30, wherein said lipid (A) is phosphatidylethanolamine.

33. The pharmaceutical composition of claim 27, 28, or 30, wherein said lipid (A) is **cholesterol**.

34. The pharmaceutical composition of claim 27, 28, or 30, wherein said lipid (A) is a phospholipid.

35. The pharmaceutical composition of claim 27, 28, or 30, wherein said lipid (A) is a sphingolipid.

36. The pharmaceutical composition of claim 27, 28, or 30, wherein said lipid (A) is a sterol.

37. A method for selectively inducing antibody production comprising administering to a host susceptible of treatment the immunogenic composition of claim 1, 18, 19, or 20.

38. A method for selectively inducing antibody production comprising administering to a host susceptible of treatment the immunogenic composition of claim 3, 21, 22, or 23.

39. The method of claim 38, wherein said first hybrid peptide comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

40. A method for selectively inducing antibody production comprising administering to a host susceptible of treatment the immunogenic composition of claim 14, 24, 25, or 26.

41. The method of claim 40, wherein said first hybrid peptide in said

immunogenic composition (III), of (I), comprises said hydrophilic peptide (2)(a) covalently bound to said amphipathic peptide (1).

42. The method of claim 37, wherein said lipid (A) is phosphatidylethanolamine.

43. The method of claim 38, wherein said lipid (A) is phosphatidylethanolamine.

44. The method of claim 40, wherein said lipid (A) is phosphatidylethanolamine.

45. The method of claim 29, wherein said lipid (A) is **cholesterol**.

46. The method of claim 38, wherein said lipid (A) is **cholesterol**.

47. The method of claim 40, wherein said lipid (A) is **cholesterol**.

AI US 1993-160093 19931201 (8) <--

DRWD . . . Woods analysis of peptides useful in the immunogenic composites of the present invention: FIG. 2 shows profiles for amphipathic peptides, **HIV**(469-511) and **HIV**(487-511) and for hydrophilic peptides **HIV**(469-485) and **HIV**(500-511); FIG. 3 shows a profile for the amphipathic peptide **HIV**(578-608); FIG. 4 shows a profile for the hydrophilic peptide **HIV**(647-659); and FIG. 5 shows a profile for the neutral peptide $(NANP)_n$. The asterisks represent the degree of hydrophilicity or hydrophobicity,. . .

DETD . . . by measuring the ability of the peptide to promote the release of carboxyfluorescein from the internal aqueous space of preformed **liposomes** as described below. However, not all amphipathic peptides will necessarily perturb the integrity of a phospholipid membrane, and, accordingly, for. . .

DETD . . . by measuring the ability of the peptide to promote the release of carboxyfluorescein from the internal aqueous space of preformed **liposomes** (Szoka and Papahadjopoulos, Proc. Nat. Acad. Sci. USA 75:4194-4198 (1978) and Weinstein, J. N. et al., Science 195:489-491 (1977)), although. . .

DETD . . . can be determined by measuring emission at 520 nm 30 minutes after the addition of peptide to a suspension of **liposomes**. In general, an increase in fluorescence greater than 5% of the total possible increase is indicative of amphipathicity. Total possible. . .

DETD Examples of amphipathic peptides include the following peptides derived from the human immuno deficiency virus, **HIV**, envelope protein: **HIV**(487-511), **HIV**(469-511) and **HIV**(578-608) (Starcich et al. (Cell 45:637-648 (1986)). Further, antibodies to the following sequences recognize the **HIV** surface glycoprotein or the sequences are included in a region to which neutralizing antibodies are produced: **HIV**(735-752)(D-R-P-E-G-I-E-E-E-G-G-E-R-D-R-S-NH_2, Kennedy et al., Science 231:1556-1559 (March 1986)), **HIV**(340-368)(N-N-T-L-K-Q-I-D-S-K-L-R-E-Q-F-G-N-N-L-Q-S-S-G-C-NH_2) alone or **HIV**(299-329)(H-R-P-N-N-N-T-R-K-I-R-I-E-R-E-P-E-R-A-E-K-I-E-N-M-R-Q-C-NH_2) with an amphipathic peptide of **HIV** (e.g. 487-511) to enhance antibody production. Both **HIV**(340-368) and **HIV**(299-329) are from a region of the virus which produces neutralizing antibodies. (Putney et al., Science 234:1392-1395 (December 1986)).

DETD The primary sequences of **HIV**(487-511), **HIV**(469-511) and **HIV**(578-608) are set forth in FIG. 1.

DETD Hopp and Woods profiles of **HIV**(487-511) and **HIV**(469-511) are shown in FIG. 2 and a Hopp and Woods profile of **HIV**(578-608) is shown in FIG. 3.

DETD Examples of suitable lipids include phospholipids such as phosphatidylethanolamine (PE); sterols such as **cholesterol**; spingolipids such as sphingomyelin; glycolipids such as myeline; and other diacyl containing lipid structures, e.g., diacylamines.

DETD In the second method the solution containing **unilamellar** vesicles is

prepared in the same manner as the particulate suspension of vesicles is prepared in the first method. Peptide. . .

DETD Examples of lipids useful as the lipid component of the lipid/sterol mixture include phosphatidylserine (PS), **phosphatidylcholine** (PC), spingomyelin (SP), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG); phosphatidic acid (PA), and cardiolipin.

DETD Examples of sterols that can be used in the mixture include **cholesterol** and lanosterol.

DETD An especially preferred sterol is **cholesterol**.

DETD Examples of hydrophilic peptides include **HIV**(469-485), **HIV**(500-511) and **HIV**(647-659).

DETD Hopp and Woods profiles of **HIV**(469-485) and **HIV**(500-511) are shown in FIG. 2 and a Hopp and Woods profile of **HIV**(647-659) is shown in FIG. 4.

DETD . . . (Cease, et al., Proc. Natl. Acad. Sci. U.S.A. 84:4249-4253 (1987)) are useful in conjunction with hydrophilic or neutral peptides from **HIV**(e.g. **HIV**(299-329)) for inducing an immune response to these non-immunigenic peptides.

DETD Examples of hybrid peptides include: (a) **HIV**(469-511), comprised of the non-immunogenic hydrophilic peptide **HIV**(469-485) and the non-immunogenic amphipathic peptide **HIV**(487-511), and (b) **HIV**(487-511), comprised of the non-immunogenic hydrophilic peptide **HIV**(500-511) and the nonimmunogenic amphipathic peptide **HIV**(487-499).

DETD When **HIV**(469-511) is made part of an immunogenic composite according to the present invention antibodies are produced against the non-immunogenic hydrophilic peptide **HIV**(469-485) (as well as against the peptides **HIV**(487-511), (469-511), and (500-511)).

DETD When **HIV**(487-511) is made part of an immunogenic composite according to the present invention, antibodies are produced against the non-immunogenic hydrophilic peptide **HIV**(500-511) (as well as against the peptides **HIV**(487-511) and (469-511)).

DETD . . . sterols having associated therewith one peptide-lipid complex wherein the peptide is an amphipathic peptide such as T_2 or T_1 from **HIV** and another peptide-lipid complex wherein the peptide is a hydrophilic peptide such as **HIV**(299-329), will induce antibodies not only to the amphipathic peptides T_2 or T_1, but also to the hydrophilic peptide **HIV**(299-329). If another peptide-lipid complex is included, antibodies to the peptide(s) in the additional complex will be induced.

DETD Examples of clinical uses include the preparation of vaccines and induction of systemic or **mucosal** antibodies against sperm for use as a contraceptive.

DETD The dosage form can be **oral**, nasal, intramuscular, intravenous, intraperitoneal, intraocular, subcutaneous, intravaginal, or on any **mucosal** surface.

DETD 1. The amphipathic peptide **HIV**(487-511), **HIV**(469-511), or **HIV**(578-608), prepared by solid-phase procedures (Yarney and Marrifield. In "The Peptide: Analysis, Synthesis, Biology." Academic Press, N.Y. Vol. 2 1-284.) was. . .

DETD III. Preparation of immunogenic composites comprising peptide-lipid complex associated with a mixture of lipids and a sterol (**cholesterol**)

DETD A. Lipids--MPB-PE, sphingomyelin (SP), **phosphatidylcholine** (PC), phosphatidylserine (PS).

DETD Sterol--**cholesterol** (Ch).

DETD 1. The lipids and **cholesterol** (Ch) were dissolved in ether (10 mg/ml) at a molar ratio of MPB-PE:SP:PC:PS:Ch of 2:1:1:1:5.

DETD . . . mM citric acid, 35 mM disodium phosphate, 108 mM NaCl, 1 mM EDTA, pH 4.5) at 4 mg lipid and **cholesterol**/ml.

DETD 5. The immunogenic composite comprising the protein-lipid complex associated with a mixture of lipids and **cholesterol** was recovered from the dialysis bag and stored refrigerated (4° C.).

DETD . . . peptide, and then covalently bonding the reduced hybrid peptide to a derivatized lipid present in solution with other lipids and **cholesterol** as described above in Example 1 and dialyzing as described in Example 1 to form an association of the hybrid peptide-lipid complex

DETD . . . neutral or hydrophilic peptide and a reduced amphipathic peptide to a derivatized lipid present in solution with other lipids and **cholesterol** as described in Example 1, and dialyzing as described in Example 1 to form an association of both peptide-lipid complexes. . .

DETD Using method 1, immunogenic composites were prepared using the **HIV** peptides (487-511), (469-511) and (578-608).

DETD . . . in Example 1 but where the peptide was $(NANP)_3$, IL-2(1-12)-trans (the primary amino acid sequence is shown in FIG. 1), **HIV** env(469-485), **HIV** env(500-511) and **HIV** env(578-608) all of which are either hydrophilic or neutral). Each was used in an amount equivalent to 30 μg of. . .

DETD . . . Immunized With Various Peptide-Lipid Conjugates

Peptide to Immunizing Peptide	Log_{10} Antibody Titer
Hydrophilic or Neutral	
$(NANP)_3$	0.0(6)*
IL-2(1-12)-trans	1.2 ± 1.3(6)
HIV env. (469-485)	0.0(5)
HIV env. (500-511)	0.0(5)
HIV env. (647-659)	0.0(5)
Amphipathic	
HIV env. (469-511)	4.4 ± 0.5(5)
HIV env. (487-511)	4.3 ± 0.8(4)
HIV env. (578-608)	4.4 ± 0.4(5)

*the figure in parenthesis indicates the number of mice per group.

DETD The results clearly show that complexing an amphipathic peptide with phospholipid and associating it with additional phospholipid and **cholesterol** induces a significant immune response when injected into mice. This is in contrast to the results with neutral and hydrophilic. . . do not induce an immune response in mice even when covalently linked to phosopholipid and complexed with additional phospholipid and **cholesterol**.

DETD . . . were immunized with the immunogenic composites prepared in Example 2 and with composites prepared as in Example 1 but using **HIV** peptides (469-485) and (500-511). Antibody titers were determined as in Example 3.

DETD TABLE 2

Immune Response To Nonimmunogenic Hydrophilic Peptides Of **HIV** Envelope Protein When Synthesized Contiguous With A Hydrophobic Sequence From The Same Protein

Immunizing Peptide	Log_{10} Antibody Titer to 469-485	487-511. . .

DETD . . . Thus, these regions became immunogenic when incoporated into larger amphipathic peptides, complexed to phospholipid, and associated with additional phospholipid and **cholesterol**.

DETD TABLE 3

Effect Of Addition Of Highly Immunogenic Proteins To **Liposomes** Containing Nonimmunogenic Peptide - Phospholipid Conjugates

Peptide to Immunizing Peptide	Viral Glycoproteins	Log_{10} Antibody Titer
--	Influenza	0.0* (6)**
--	Sendai. . .	

DETD . . . when neutral or hydrophilic peptides are completed to phospholipid and added to preparations containing amphipathic structures and additional phospholipid and **cholesterol**, they become immunogenic.

DETD TABLE 4

Log_{10} [illegible]

Peptide Immunizing Peptide

HIV env. (487-511) -
mixed lipid conjugate* 5.3 ± 0.2(5)
HIV env. (487-511) -
mixed lipid conjugate** 5.4 ± 0.3(5)
HIV env. (487-511) -
mixed lipid free*** 0.0(5)
HIV env. (487-511) in
sterile PBS*** 0.0(5)

Antibody titers of mice immunized with **HIV** env. (487-511) conjugated to phospholipid by the following methods:
*MPBPE was mixed with the other phospholipids and **cholesterol** in small unilamelar vesicles and reacted with peptide overnight at room temperature. The reaction mixture was dissolved with octylD-glucoside (10 mg/mg lipid) and dialyzed against CMFPBS to form a peptide phospholipid complex.
or
MPBPE and mixed phospholipids and **cholesterol were dissolved in OctylD-glucoside (10 mg octylD-glucoside/mg lipid), reacted with peptide overnight at room temperature and subsequently dialyzed against CMFPBS. . .

DETD . . . that when the peptide is complexed to phospholipid as a lipid and associated .with additional phospholipid as a lipid and **cholesterol** as a sterol, an immunogenic composite is produced. This composite is significantly immunogenic regardless of whether the initial coupling is to MPB-PE already associated in a structure with other lipid and **cholesterol** or free in solution by virtue of being dissolved in octyl-β-D-glucoside. In contrast, if the peptide is mixed with preformed lipid-**cholesterol** structures, but not complexed with phospholipid no immune response is observed. Furthermore, no immune response is observed if solubilized 487-511. . .

DETD . . . micelle concentration, such as octyl-β-D-glycoside, along with derivatized phosphatidylethanolamine (PE), a mixture of lipids comprising, for example sphingomyelin, phosphatidylserine, and **phosphatidylcholine**, and **cholesterol** as a sterol.

DETD CONSTRUCTION OF A SYNTHETIC VACCINE AGAINST **HIV** III

DETD The following non-immunogenic hydrophilic peptides of immunologic significance are known for **HIV** III: (299-329) H-R-P-N-N-N-T-R-K-I-R-I-R-E-P-E-R-A-E-K-I-E-N-M-R-Q-C-NH_2 and (735-752) D-R-P-E-G-I-E-E-E-G-G-E-R-D-R-S.

DETD The following immunologically important amphipathic peptides also from **HIV** III have recently been described by K. B. Cease (Proc. Nat. Acad. Sci. USA 84:4249-4253 (June 1987)): T_2 (HIVenv(112-124), H-E-D-I-I-S-L-W-H-Q-S-L-K) T_1 (HIVenv(428-443), L-Q-I-I-N-M-W-Q-E-V-L-A-M-X-A-NH_2) and by Putney et al. (Science 234:1392-1395 (December 1986)): (**HIV**(340-368) H-N-N-T-L-K-Q-I-D-S-K-L-R-E-Q-F-G-N-N-L-Q-S-S-G-C-NH_2).

11. The essentially pure immunogenic composition of claim 2, wherein said lipid (A) is **cholesterol**.

12. The essentially pure immunogenic composition of claim 4, wherein said lipid (A) is **cholesterol**.

16. The essentially pure immunogenic composition of claim 14, wherein said lipid (A) in said composition (I), (II), (III), (IV), or (V)(A)is **cholesterol**.

33. The pharmaceutical composition of claim 27, 28, or 30, wherein said lipid (A) is **cholesterol**.

45. The method of claim 29, wherein said lipid (A) is **cholesterol**.

46. The method of claim 38, wherein said lipid (A) is **cholesterol**.

47. The method of claim 40, wherein said lipid (A) is **cholesterol**.

L9 ANSWER 10 OF 22 USPATFULL on STN
2001:43743 Method for inducing a systemic immune response to an **HIV** antigen.
See, Jackie R., Reno, NV, United States
See, Darryl M., Laguna Niguel, CA, United States
Bio-Sphere Technology, Reno, NV, United States (U.S. corporation)
US 6207185 B1 20010327
APPLICATION: US 1997-948568 19971010 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A method is provided for inducing a systemic immune response to an antigen selected from inactivated **HIV** I and **HIV** II antigens in a mammal. The method comprises orally administering lyophilized multilaminar **liposomes** containing the antigen. The **liposomes** have a size of from 20 nm to 20 microns. The antigen-containing **liposomes** are absorbed in the Peyer's patches of the gut. Sufficient antigen-containing **liposomes** are taken up by macrophages in the Peyer's patches to induce a systemic immune response to the antigen.

CLM What is claimed is:

1. A method for stimulating a systemic immune response to an antigen selected from the group consisting of inactivated **HIV** I and **HIV** II antigens and combinations thereof in a mammal comprising: providing a lipsomal preparation comprising lyophilized **liposomes** containing at least one antigen selected from the group consisting of inactivated **HIV** I and **HIV** II antigens, wherein the **liposomes** have at least three different sizes and consist essentially of: at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 5% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 5% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyophilization, of from about 3 microns to about 20 microns; and orally administering an effective amount of the liposomal preparation to a mammal, whereby sufficient antigen containing **liposomes** are absorbed in the Peyer's patches of the gut of the mammal and are taken up by macrophages in the Peyer's patches to stimulate a systemic immune response.

2. A method as claimed in claim 1, wherein the **liposomes** are multi-lamellar before lyophilization.

3. A method as claimed in claim 1, wherein the liposomal preparation is contained within an enterically-coated capsule.

4. A method as claimed in claim 1 wherein the **liposomes** consist essentially of at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 10% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 20% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyphilization, of from about 3 microns to about 20 microns.

5. A method as claimed in claim 1 wherein the **liposomes** comprise about 10% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, about 25% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and about 65% by volume, based on the total volume of

liposomes in the preparation, large liposomes having a size, before lyopilization, of from about 3 microns to about 20 microns.

6. A method as claimed in claim 1 wherein the **liposomes** comprise at least two different antigens.

7. A preparation for **oral** administration to a mammal capable of stimulating a systemic immune response to at least one antigen selected from the group consisting of inactivated **HIV** I and **HIV** II antigens, said preparation comprising an effective amount of lyophilized antigen-containing **liposomes**, wherein the **liposomes** have at least three different sizes, before lyophilization, and consist essentially of: at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 5% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 5% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyphilization, of from about 3 microns to about 20 microns.

8. A preparation as claimed in claim 7 wherein the **liposomes** are multi-lamellar before lyophilization.

9. A preparation as claimed in claim 7 wherein the **liposome** preparation is contained within an enterically-coated capsule.

10. A preparation as claimed in claim 7 wherein the **liposomes** comprise small, medium and large **liposomes**.

11. A preparation as claimed in claim 7 wherein the **liposomes** consist essentially of at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 10% by volume, based on the total of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 20% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyophilization, of from about 3 microns to about 20 microns.

12. A preparation as claimed in claim 7 wherein the **liposomes** consist essentially of about 10% by volume, based on the total volume of **liposomes** in the prepartion, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, about 25% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and about 65% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyophilization, of from about 3 microns to about 20 microns.

13. A preparation as claimed in claim 7 wherein the **liposomes** comprise at least two different antigens.

14. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

15. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

16. A method according to claim 1, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

17. A method according to claim 1, wherein the medium **liposomes** have a

size, before lyophilization, of about 2 microns.

18. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

19. A method according to claim 1, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

20. A method according to claim 4, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

21. A method according to claim 4 wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

22. A method according to claim 4, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

23. A method according to claim 4, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

24. A method according to claim 4, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

25. A method according to claim 4, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

26. A method according to claim 5, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

27. A method according to claim 5, wherein the large **liposomes** have a size, before lyphilization, of from about 3 microns to about 5 microns.

28. A method according to claim 5, wherein the small **liposomes** have a size, before liphilization, of about 1 micron.

29. A method according to claim 5, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

30. A method according to claim 5, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

31. A method according to claim 5, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

32. A prepairation according to claim 7, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

33. A preparation according to claim 7, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

34. A preparation according to claim 7, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

35. A preparation according to claim 7, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

have a size, before lyophilization, of about 10 microns.

37. A preparation according to claim 7, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns and the large **liposomes** have a sizes before lyophilization, of about 10 microns.

38. A preparation according to claim 11, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

39. A preparation according to claim 11, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

40. A preparation according to claim 11, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

41. A preparation according to claim 11, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

42. A preparation according to claim 11, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

43. A preparation according to claim 11, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

44. A preparation according to claim 12, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

45. A preparation according to claim 12, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

46. A preparation according to claim 12, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

47. A preparation according to claim 12, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

48. A preparation according to claim 12, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

49. A preparation according to claim 12, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

50. A method according to claim 1 wherein the antigen containing **liposomes** are capable of being absorbed in the Peyer's patches of the gut of the mammal and are capable of being taken up by macrophages in the Peyer's patches to stimulate a systemic immune response without the presence of an adjuvant.

51. A method according to claim 1 wherein the antigen containing **liposomes** are capable of being absorbed in the Peyer's patches of the gut of the mammal and are capable of being taken up by macrophages in the Peyer's patches to stimulate a systemic immune response without generating a typical adjuvant effect.

TI Method for inducing a systemic immune response to an **HIV** antigen

AI US 1997-948568 19971010 (8) <--

AB A method is provided for inducing a systemic immune response to an antigen selected from inactivated **HIV** I and **HIV** II antigens in a mammal. The method comprises orally administering lyophilized multilaminar **liposomes** containing the antigen. The **liposomes** have a size of from 20 nm to 20 microns. The antigen-containing **liposomes** are absorbed in the Peyer's patches of the gut. Sufficient antigen-containing **liposomes** are taken up by macrophages in the Peyer's patches to induce a systemic immune response to the antigen.

SUMM This invention relates to a method for inducing a systemic immune response to an **HIV** antigen and more particularly to vaccines suitable for **oral** administration.

SUMM . . . surfaces of the body serve as a barrier to antigenic material. However, those surfaces are by no means impenetrable. The **mucosal** immune system provides the next major line of defense against a majority of human pathogens. The **mucosal** immune system includes gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue, the salivary glands, the conjunctiva, the mammary gland, parts of. . .

SUMM Childers et al. (**Oral** Microbiol. Immunol. 1994:9:146-153) reported that lyophilized **liposomes** containing S. mutans antigen can be administered orally to human patients and will be absorbed by GALT to elicit a. . .

SUMM Traditionally, to obtain a systemic immune response by **oral** administration of an antigen, it was required that the antigen be associated with an adjuvant. The presence of the adjuvant. . .

SUMM A need therefore exists to induce a systemic immune response by **oral** administration without the presence of an adjuvant and without inducing the above-described adjuvant effects, but instead by uptake by the. . .

SUMM . . . in a mammal and does not require the presence of an adjuvant. According to the present invention, the lyophilized antigen-containing **liposomes** do not directly target the CD4 cells and cytotoxic lymphocytes, but instead are taken up by the macrophages in the. . . that occurs when an antigen/adjuvant combination is orally administered. Thus, the present invention involves a method whereby the antigen containing **liposomes** can be orally administered without an adjuvant to induce a systemic immune response. Moreover, the inventive methods do not generate. . .

SUMM The inventive method comprises first incorporating at least one antigen selected from inactivated **HIV** I and **HIV** II antigens into **liposomes**, preferably multilamellar **liposomes** having a size from about 20 nm to about 20 microns or greater, preferably from about 200 nm to about 10 microns and more preferably from about 1 micron to about 5 microns. The antigen-containing **liposomes** are then lyophilized and packaged in a suitable form, such as a pill or capsule, for **oral** ingestion. Means, such as an enteric coating are provided for preventing breakdown of the preparation in the stomach but allowing. . . in the gut, i.e., small intestine. Once orally ingested, the preparation passes through the stomach into the gut wherein antigen-containing **liposomes** are absorbed in the Peyer's patches of the gut. In the Peyer's patches, sufficient antigen-containing **liposomes** are taken up by macrophages to induce a systemic immune response and preferably a long-term systemic immune response to the. . .

SUMM The invention further provides a preparation suitable for **oral** ingestion for inducing a systemic response, and preferably a long-term systemic immune response, to one or more antigens selected from inactivated **HIV** I and **HIV** II antigens. The composition comprises lyophilized, preferably multilamellar, **liposomes** that contain the antigen(s). The **liposomes** have a size, before lyophilization, of from about 20 nm to about 20 microns or greater, preferably from about 200. . . nm to about 10 microns, and more preferably from about one to about five microns. A particularly preferred composition comprises **liposomes** of varying sizes including small **liposomes**, i.e., about 20 mn to about 1 micron, medium **liposomes**, i.e., about 1 to about 3

microns, and large **liposomes**, i.e. about 5 to about 20 microns or greater and preferably about 3 to about 5 microns. It is presently preferred that such a composition comprise at least 5% by volume small **liposomes**, at least 10% by volume medium **liposomes**, and at least 20% by volume large **liposomes**. The composition preferably comprises means for preventing breakdown of the preparation in the stomach but for allowing digestion of the **liposomes** in the gut. In the gut, the **liposomes** are absorbed by Peyer's patches and sufficient **liposomes** are taken up by macrophages to stimulate a long term systemic immune response.

DRWD FIG. 1 is an electron photomicrograph (magnification: 100,000×) of **liposomes** in lymphoid tissue of a Peyer's patch.

DRWD FIG. 8 is an electron photomicrograph (magnification: 10,000×) of **liposomes** surrounding a white blood cell in a venule of the Peyer's patch.

DRWD FIG. 10 is an electron photomicrograph (magnification: 40,000×) showing **liposomes** at the cellular membrane and inside a macrophage in the Peyer's patch.

DRWD FIG. 11 is an electron photomicrograph (magnification: 70,000×) showing **liposomes** inside a macrophage in the Peyer's patch.

DRWD FIG. 12 is an electron photomicrograph (magnification: 75,000×) showing **liposomes** adhering to a venule wall in the lymphoid cells of the Peyer's patch.

DRWD FIG. 13 is an electron photomicrograph (magnification 25,000×) showing 980 nm **liposomes** in a macrophage vacuole 7 days after **oral** inoculation.

DRWD FIG. 14 is an electron photomicrograph (magnification 12,500×) showing 10 micron **liposomes** in a macrophage 21 days after **oral** inoculation.

DRWD FIG. 15 is an electron photomicrograph (magnification 40,000×) showing 2 micron **liposomes** in a macrophage vacuole 60 days after **oral** inoculation.

DETD . . . the macrophage of the GALT for processing to confer systemic immunity, and this is dependent upon the size of the **liposome** presented to the GALT.

DETD Size and composition of the **liposomes** are important in determining the duration of the systemic immune response to the incoiporated antigen. Administration of **liposomes** of varying size and composition ensure a long lasting immune response, and thus avoid the need for repéated vaccine administrations.. . .

DETD It has been found that **liposomes** containing one or more antigens and having a particular size from about 20 nm to about 20 microns or greater,. . . liposomal antigen in the Peyer's patches (outside of the macrophages) initiates a local immune response to the antigen as the **liposomes** breakdown and release the antigen. The uptake of sufficient liposomal antigen in the macrophages stimulates a systemic immune response, and preferably a long-term systemic immune response, to the antigen(s) as the **liposomes** breakdown within the macrophages to release antigen.

DETD As used herein, "local immune response" refers to **mucosal** IgA, which confers protection from organisms in the bowel lumen and is characterized by secretion of local sIgA.

DETD . . . used herein, "sufficient liposomal antigen to stimulate a systemic immune (or long-term systemic immune) response" means that amount of antigen-containing **liposomes** that affect a detectible systemic immune response (or long-term systemic immune response). A systemic immune response may be confirmed by. . .

DETD . . . the invention, the antigen is an attenuated or killed microorganism, such as a virus or bacteria, rendering the preparation an **oral** vaccine against that microorganism.

DETD As used herein, "inactivated **HIV** I and **HIV** II antigens" include any substance that, when introduced into a mammal, will induce a detectable immune response, both humoral and cellular. Typical **HIV** I and **HIV** II antigens include but are not limited to, p24 antigen, gp120, gp41, and envelope proteins.

DETD The **liposomes** of the present invention may be made of any suitable

phospholipid, glycolipid, derived lipid, and the like. Examples of suitable. . . the like. Suitable derived lipids include cholic acid, deoxycholic acid, and the like. The presently preferred lipid for forming the **liposomes** is egg **phosphatidylcholine**.

DETD The **liposomes** may be formed by any of the known methods for forming **liposomes** and may be loaded with antigen according to known procedures. Known methods for forming liposomal antigen are described, for example, in U.S. Pat. No. 4,235,871 to Papahadjopoulos, et al., and **Oral** Microbiology and Immunology, 1994, 9:146-153, the disclosures of which are incorporated herein by reference. What is formed is an emulsion. . .

DETD Viral, bacterial and parasitic antigens may all be incorporated into **liposomes** and generate long-term immunity. In all cases, varying the size of the **liposome** for each antigen is crucial. The antigens may first be individually incorporated into **liposomes** and then given individually or mixed with **liposomes** containing other antigens. Viral, bacterial and/or parasitic antigens may be combined. In an exemplary embodiment of the invention, the **liposomes** are loaded with p24 antigens. The **liposomes** may also be loaded with other **HIV** antigens or whole virus.

DETD It is also understood that rather than loading multiple viral antigens into each **liposome**, preparations may be prepared comprising a mixture of **liposomes** wherein each **liposome** contains only a single antigen. If desired, the **liposomes** may be loaded with a therapeutic drug in addition to the antigen.

DETD It is preferred that the **liposomes** used in the present invention have an average mean diameter from about 20 nm to about 20 microns, preferably from. . .

DETD **Liposomes** larger than about 20 microns are generally not preferred because they tend not to be taken up by the macrophages and only affect a local secretory antibody response. That is, the presence of large antigen-containing **liposomes** in the lymphoid tissue of the Peyer's patches will induce gut-associated lymphoid tissue (GALT) to produce IgA antibodies to destroy. . .

DETD **Liposomes** smaller than about 20 nm are generally not preferred because they also tend not to be processed adequately by macrophages. These smaller **liposomes** tend to reside in the lymphoid tissue until they eventually are absorbed into the bloodstream and are destroyed by the reticuloendothelial (RE) system. The smaller **liposomes** may induce a low grade production of secretory IgA, but do not stimulate systemic immunity.

DETD It has been found that antigen-containing **liposomes** of from about 20 nm to about 20 microns, preferably from about 200 nm to about 10 microns and more. . . about 1 micron to 5 microns tend to be absorbed by macrophages in the Peyer's patches. The macrophages digest the **liposomes** to release the antigen, which is then presented or displayed at the surface of the macrophage. The macrophages act as. . .

DETD It is preferred that the **liposomes** be a mixture of sizes. Such heterogeneous sizes of **liposomes** are preferred as they are broken down over a period of time, e.g., up to 180 days or more by the macrophages. Preferably, the mixture of sizes will include **liposomes** having a size of about 20 nm to about 1 micron (small **liposomes**), **liposomes** having a size of about 1 micron to about 3 microns (medium **liposomes**) and **liposomes** having a size of about 3 to about 20 microns (large **liposomes**). Preferred large **liposomes** are those having a size of from about 3 to about 5 microns. Preferably, there is at least about 5% by volume of each size of **liposomes**, i.e., small, medium and large, in the composition. More preferably, there is at least about 5% by volume of small **liposomes**, at least 10% by volume medium **liposomes**, and at least 20% by volume large **liposomes**. A particularly preferred composition comprises about 10% by volume small **liposomes**, about 25% by volume medium **liposomes** and about 65% by volume large **liposomes**.

DETD In a composition containing a heterogeneous population of **liposomes**, there may be a uniform distribution of sizes or two or more discrete, homogeneous populations. A combination of small, medium. . .

DETD Compositions comprising **liposomes** of various sizes allow antigens to

be released in the macrophages over a long period of time, thereby continuing to stimulate a systemic immune response over a period of time. The small size **liposomes** are taken up by the macrophages quickly and provide an immediate systemic immune response. Medium size **liposomes** are taken up by the macrophages, but at a slower pace. These **liposomes** act as a booster, i.e., provide an amnestic response. The larger size **liposomes** take even longer to be taken up by the macrophages and act as a second booster, i.e., provide a second amnestic response. Hence, use of **liposomes** of varying sizes enables a single dose of the antigen-containing **liposomes** to be sufficient to result in long term, and even permanent, immunity to the antigen.

DETD The **liposomes** may be **unilamellar** or multilamellar. Production of **unilamellar** and multilamellar **liposomes** is also well known in the art and is described, for example, in U.S. Pat. No. 5,008,050 to Cullis et. . .

DETD . . . to 20 micron pore size, the filter being either the straight path or tortuous path type. Other methods of treating **liposomes** to form a homogenous size distribution are ultrasonic exposure, the French press technique, hydrodynamic shearing, homogenization using, for example, a. . .

DETD In a particularly preferred embodiment of the invention, the **liposomes** are passed one to ten and preferably 4 times through an M-110 Series Laboratory Microfluidizer manufactured by Microfluidics Corporation at a pressure of, e.g., 14,000 pounds per square inch to achieve a generally homogenous population of **liposomes** having an average mean diameter of about 1 micron. **Liposomes** of other sizes can be prepared using the same method by adjusting the number of runs through the microfluidizer, the. . .

DETD In sonication techniques, the raw materials for the **liposomes**, e.g., phospholipids, are combined with antigens, placed in a sonicator, and sonicated for a time, at a temperature and at a speed sufficient to obtain **liposomes** of the desired size. For example, in a particularly preferred method, raw materials are placed in a Brinkman Inc. or. . . to 10,000 meters per second at 50° C. for 20, 5 and 2 minutes to obtain small, medium and large **liposomes**, respectively. Typically, larger sonication times result in smaller **liposomes**.

DETD . . . lyophilized. Lyophilized liposomal antigen can be stored at room temperature for one half to three years without degradation of the **liposomes** or antigen.

DETD . . . disclosure of which is incorporated herein by reference. Lyophilization procedures preferably include the addition of a drying protectant to the **liposome** suspension. The drying protectant stabilizes the **liposome** suspension. The drying protectant stabilizes the **liposomes** so that the size and content are maintained during the drying procedure and through rehydration. Preferred drying agents are saccharide. . .

DETD The lyophilized liposomal antigen may be packaged for **oral** administration in either a pill form or a capsule. An enteric coating is preferably applied to the liposomal antigen to. . .

DETD To establish the effective absorption of lyophilized **liposomes** by Peyer's patches and uptake by macrophages, the following protocol was followed:

DETD Preparation of antigen-containing **liposomes**:

DETD Antigen-containing **liposomes** having a diameter of approximately 142 nanometers used in the experimental study described below were prepared according to the following. . .

DETD 3. In another beaker 10.59 gms of egg **phosphatidylcholine** (EPC) (Sigma) is combined with 8.38 ml of ethanol (anhydrous, Sigma E3884) and mixed until dissolved. To this add 67.5. . .

DETD 100 micrograms of lyophilized **liposomes** 142 nanometers in diameter were suspended in 0.3 ml of 0.5% xanthum gum aqueous solution. The mixture was given via. . .

DETD . . . gut were sectioned and slides were prepared. Photomicrographs were taken and are presented here as FIGS. 1-12. The photomicrographs show **liposomes** (FIG. 1) residing in venules and extracellular tissue of the Peyer's patch (FIGS. 2, 6, 7, 8, 12). They also show that the

liposomes were not present in the splenic lymphoid tissue which indicate that the **liposomes** were staying in the Peyer's patches and not circulating through the blood stream in the mouse. (FIGS. 3, 4). Finally, the photomicrographs show **liposomes** being absorbed and digested by macrophages (FIGS. 5, 9, 10, 11).

DETD Viral proteins were encapsulated with 3 different particle size **liposomes** as follows: Before beginning, 3 round bottom flasks were labeled A (2 minutes), B (5 minutes) and C (20 minutes). 783 mg of diphosphatidylchoxene (DPPC) (Avanti), 180 mg **Cholesterol** (Sigma), and 36mg Dicetyl-Phosphate (Sigma) was added to each of the flasks. 25 mg N-(1-pyrene sulfonyl)-1,2 hexadecanoyl-sn-glycero-3 phosphoethanolamine, triethylammonium sale. . .

DETD The resultant 3 **liposomes** (2 um, 10 um, 908 nm) were given to male CD-1 mice weighing 16-18 g obtained from Charles Rivers Farms,. . . or mixed for either 1 2 or 3 doses over the same number of weeks. For all experiments, 30 mg **liposomes** were given orally in 0.3 cc containing sodium acetate buffer, pH 9.0. In one experiment, 120 mg of mixed **liposomes** were given. The final set of mice were given either mixed **liposomes** or a placebo and then infected with CVB5/C59.

DETD To show the success of the **liposome** vaccine in stimulating a specific antibody response in mice, serial determinations of neutralizing antibody to all six coxsackie B serogroups were made in groups of 5 mice for each **liposome** tested. Means for each **liposome** were calculated for neutralizing antibody titer in plasma obtained 8-60 days after final dose of vaccine. Eight days after final dose of **liposomes**, a modest rise in titer to all strains tested was recorded. The smallest **liposome** (909 nm) gave the largest initial response after one dose (mean 4.2+/-SD2.3) but had little increase with repeated doses. The largest (10 um) **liposome** resulted in the greatest antibody response after 3 doses but did not result in detectable antibody levels 24 days after final dose. A single dose of the mixed **liposomes** produced an antibody response still detectable 21 days after final dose.

DETD TABLE 1

Neutralizing antibody titers to 6 CVB strains after various doses of vaccine.

Liposomes	# doses	Days since last dose	Mean Neutralizing Antibody Titer
980 nm	1	8	4.2+/-2.3
	2	8	4.9+/-2.5
	3	8	4.7+/-2.7

2 um 1. . .

DETD To confirm the ability of the **oral** vaccination to limit challenge virus infection, titers of virus were determined in the pancreas of mice killed 3 days after infection. Two groups of 5 mice were used; one group was given 3 doses of mixed **liposomes** and the other group was given buffer placebo. The placebo group ended up with a mean titer of 5.3×10^4 (pfu/mg). . .

DETD As shown in FIG. 13, seven days after final **oral** inoculation, 980 nm **liposomes** are visible in vacuoles within macrophages of the Peyer's Patches. As shown in FIG. 14, 21 days after **oral** inoculation, a 10 micron **liposome** was observed in a macrophage of the Peyer's patches. As shown in FIG. 15, 60 days after **oral** inoculation, 2 micron **liposomes** were observed in a vacuole within a macrophage of the Peyer's Patches.

DETD p24 antigen was encapsulated in 3 different particle size **liposomes** as follows: A solution of PyS DHPE was prepared in a test tube by dissolving 25 mg of PyS DHPE. . . chloroform. Lipid solution was then prepared in a separate test tube by combining 313 mg of DPPC, 72 mg of **cholesterol**, 14 mg of dicetylphosphate, 144 μL PyS DHPE solution, and 1.056 ml of chloroform, for a total volume of approximately. . .

DETD . . . first tube was warmed at 50° C. for 2 minutes then sonicated at 50° C. for 2 minutes to obtain **liposomes** have a diameter of approximately 5 μm. The solution in the second tube was warmed at 50° C. for 2 minutes then sonicated at 50° C. for 5 minutes to obtain **liposomes** having a diameter of approximately 2 μm. The solution in the third test tube was warmed at 50° C.

[illegible] μm. Approximately 10 μl of **liposomes** was removed from each test tube for particle sizing. The **liposomes** from all three test tubes were combined for a total volume of approximately 15 ml. The solution was aliquoted into. . .

DETD The resultant lyophilized **liposome** mixtures of three different-sized **liposomes** (1 μm, 2 μm, and 5 μm) were orally administered to male CD-1 mice weighing 16-18 g obtained from Charles Rivers Farms, Wilmington, Mass. For all experiments, 30 mg **liposome** mixtures were orally administered in 0.3 cc containing sodium acetate buffer, pH 9.0, so that each mouse received 60 μg. . .

DETD To show the success of the **liposome** vaccine in stimulating a specific antibody response in mice, EIA assays to p24 antigen were conducted at weekly intervals for. . .

DETD By the second week after vaccination, **liposomes** were seen in macrophages of the Peyer's patches.

1. A method for stimulating a systemic immune response to an antigen selected from the group consisting of inactivated **HIV** I and **HIV** II antigens and combinations thereof in a mammal comprising: providing a lipsomal preparation comprising lyophilized **liposomes** containing at least one antigen selected from the group consisting of inactivated **HIV** I and **HIV** II antigens, wherein the **liposomes** have at least three different sizes and consist essentially of: at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 5% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 5% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyophilization, of from about 3 microns to about 20 microns; and orally administering an effective amount of the liposomal preparation to a mammal, whereby sufficient antigen containing **liposomes** are absorbed in the Peyer's patches of the gut of the mammal and are taken up by macrophages in the. . .

2. A method as claimed in claim 1, wherein the **liposomes** are multi-lamellar before lyophilization.

4. A method as claimed in claim 1 wherein the **liposomes** consist essentially of at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 10% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 20% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyphilization, of from about 3 microns to about 20 microns.

5. A method as claimed in claim 1 wherein the **liposomes** comprise about 10% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, about 25% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and about 65% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyopilization, of from about 3 microns to about 20 microns.

6. A method as claimed in claim 1 wherein the **liposomes** comprise at least two different antigens.

7. A preparation for **oral** administration to a mammal capable of stimulating a systemic immune response to at least one antigen selected from the group consisting of inactivated **HIV** I and **HIV** II antigens, said preparation comprising an effective amount of lyophilized

antigen containing **liposomes**, wherein the **liposomes** have at least three different sizes, before lyophilization, and consist essentially of: at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 5% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 5% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyphilization, of from about 3 microns to about 20 microns.

8. A preparation as claimed in claim 7 wherein the **liposomes** are multi-lamellar before lyophilization.

9. A preparation as claimed in claim 7 wherein the **liposome** preparation is contained within an enterically-coated capsule.

10. A preparation as claimed in claim 7 wherein the **liposomes** comprise small, medium and large **liposomes**.

11. A preparation as claimed in claim 7 wherein the **liposomes** consist essentially of at least 5% by volume, based on the total volume of **liposomes** in the preparation, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, at least 10% by volume, based on the total of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and at least 20% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyophilization, of from about 3 microns to about 20 microns.

12. A preparation as claimed in claim 7 wherein the **liposomes** consist essentially of about 10% by volume, based on the total volume of **liposomes** in the prepartion, small **liposomes** having a size, before lyophilization, of from about 20 nm to about 1 micron, about 25% by volume, based on the total volume of **liposomes** in the preparation, medium **liposomes** having a size, before lyophilization, of from about 1 micron to about 3 microns, and about 65% by volume, based on the total volume of **liposomes** in the preparation, large **liposomes** having a size, before lyophilization, of from about 3 microns to about 20 microns.

13. A preparation as claimed in claim 7 wherein the **liposomes** comprise at least two different antigens.

14. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

15. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

16. A method according to claim 1, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

17. A method according to claim 1, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

18. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

19. A method according to claim 1, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

20. A method according to claim 1, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

21. A method according to claim 4 wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

22. A method according to claim 4, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

23. A method according to claim 4, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

24. A method according to claim 4, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

25. A method according to claim 4, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

26. A method according to claim 5, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

27. A method according to claim 5, wherein the large **liposomes** have a size, before lyphilization, of from about 3 microns to about 5 microns.

28. A method according to claim 5, wherein the small **liposomes** have a size, before liphilization, of about 1 micron.

29. A method according to claim 5, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

30. A method according to claim 5, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

31. A method according to claim 5, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

32. A prepairation according to claim 7, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

33. A preparation according to claim 7, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

34. A preparation according to claim 7, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

35. A preparation according to claim 7, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

36. A preparation according to claim 7, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

37. A preparation according to claim 7, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns and the large **liposomes** have a sizes before lyophilization, of about 10 microns.

38. A preparation according to claim 11, wherein the large **liposomes**

have a size, before lyophilization, of from about 3 microns to about 10 microns.

39. A preparation according to claim 11, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

40. A preparation according to claim 11, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

41. A preparation according to claim 11, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

42. A preparation according to claim 11, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

43. A preparation according to claim 11, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

44. A preparation according to claim 12, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 10 microns.

45. A preparation according to claim 12, wherein the large **liposomes** have a size, before lyophilization, of from about 3 microns to about 5 microns.

46. A preparation according to claim 12, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron.

47. A preparation according to claim 12, wherein the medium **liposomes** have a size, before lyophilization, of about 2 microns.

48. A preparation according to claim 12, wherein the large **liposomes** have a size, before lyophilization, of about 10 microns.

49. A preparation according to claim 12, wherein the small **liposomes** have a size, before lyophilization, of about 1 micron, the medium **liposomes** have a size, before lyophilization, of about 2 microns, and the large **liposomes** have a size, before lyophilization, of about 10 microns.

50. A method according to claim 1 wherein the antigen containing **liposomes** are capable of being absorbed in the Peyer's patches of the gut of the mammal and are capable of being. . .

51. A method according to claim 1 wherein the antigen containing **liposomes** are capable of being absorbed in the Peyer's patches of the gut of the mammal and are capable of being. . .

=> d his

(FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004)

FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004

L1	33450	S	(HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L2	3069	S	L1 AND (ALUMINUM HYDROXIDE)
L3	1785	S	L1 AND (UNILAMELLAR)
L4	1772	S	L3 AND LIPOSOME?
L5	434	S	L4 AND (PHOSPHATIDYLCHOLINE)
L6	402	S	L5 AND CHOLESTEROL
L7	107	S	L6 AND MUCOSAL
L8	83	S	L7 AND ORAL

=> s l1 and liposome?
         42761 LIPOSOME?
L10   13577 L1 AND LIPOSOME?

=> s l10 and liposome?/clm
          3822 LIPOSOME?/CLM
L11   897 L10 AND LIPOSOME?/CLM

=> s l11 and unilamellar
          5574 UNILAMELLAR
L12   244 L11 AND UNILAMELLAR

=> s l12 and unilamellar/clm
           258 UNILAMELLAR/CLM
L13   18 L12 AND UNILAMELLAR/CLM

=> d l13,cbib,1-18

L13 ANSWER 1 OF 18 USPATFULL on STN
2004:57947 **Liposomes.**
    Gregoriadis, Gregory, London, UNITED KINGDOM
    US 2004043954 A1 20040304
    APPLICATION: US 2003-617734 A1 20030714 (10)
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 2 OF 18 USPATFULL on STN
2003:13075 Vaccine comprising an iscom consisting of sterol and saponin which
    is free of additional detergent.
    Friede, Martin, Farnham, UNITED KINGDOM
    Garcon, Nathalie, Wavre, BELGIUM
    SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S.
    corporation)
    US 6506386 B1 20030114
    WO 2000007621 20000217
    APPLICATION: US 2001-744800 20010604 (9)
    WO 1999-EP5587 19990803
    PRIORITY: GB 1998-17052 19980805
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 3 OF 18 USPATFULL on STN
2002:275943 Expression of cloned genes in the lung by aerosol and
    liposome-based delivery.
    Debs, Robert James, Mill Valley, CA, United States
    Zhu, Ning, San Francisco, CA, United States
    The Regents of the University of California, Oakland, CA, United States
    (U.S. corporation)
    US 6468798 B1 20021022
    APPLICATION: US 1998-6841 19980114 (9)
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 4 OF 18 USPATFULL on STN
2002:213456 Materials and methods for making improved **liposome** compositions.
    Onyuksel, Hayat, Western Springs, IL, UNITED STATES
    Rubinstein, Israel, Highland Park, IL, UNITED STATES
    US 2002114829 A1 20020822
    APPLICATION: US 2001-995263 A1 20011127 (9)
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 5 OF 18 USPATFULL on STN
2002:136589 Archaesomes, archaeosomes containing coenzyme Q10 and other types

of **liposomes** containing coenzyme Q10 adjuvants and as delivery vehicles.
Sprott, G. Dennis, Orleans, CANADA
Patel, Girishchandra B., Nepean, CANADA
Makabi-Panzu, Boby, Gatineau, CANADA
National Research Council of Canada, Ottawa, CANADA (non-U.S. corporation)
US 6403117 B1 20020611
APPLICATION: US 2000-612618 20000706 (9)
PRIORITY: US 1995-8724P 19951215 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 6 OF 18 USPATFULL on STN
2001:233138 Vaccines.
Friede, Martin, Cardiff, CA, United States
Garcon, Nathalie, Rixensart, Belgium
SmithKline Beecham Biologicals s.a. (U.S. corporation)
US 2001053365 A1 20011220
APPLICATION: US 2001-819464 A1 20010328 (9)
PRIORITY: GB 1995-8326 19950425
GB 1996-910019 19960401
GB 1996-20795 19961005
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 7 OF 18 USPATFULL on STN
2000:137879 Archaeosomes, archaeosomes containing coenzyme Q_{10}, and other types of **liposomes** containing coenzyme Q_{10} as adjuvants and as delivery vehicles.
Sprott, G. Dennis, Orleans, Canada
Patel, Girishchandra B., Nepean, Canada
Makabi-Panzu, Boby, Gatineau, Canada
Tolson, Douglas L., Victoria, Canada
National Research Council of Canada, Ottawa, Canada (non-U.S. corporation)
US 6132789 20001017
WO 9722333 19970626
APPLICATION: US 1998-77956 19980612 (9)
WO 1996-CA835 19961213 19980612 PCT 371 date 19980612 PCT 102(e) date
PRIORITY: US 1995-8724P 19951215 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 8 OF 18 USPATFULL on STN
2000:53722 Cationic lipids and the use thereof.
Unger, Evan C., Tucson, AZ, United States
Shen, Dekang, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
Imarx Pharaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 6056938 20000502
APPLICATION: US 1998-73181 19980505 (9)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 9 OF 18 USPATFULL on STN
1999:159514 Stabilized compositions of fluorinated amphiphiles for methods of therapeutic delivery.
Unger, Evan C., Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5997898 19991207
APPLICATION: US 1995-465868 19950606 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 10 OF 18 USPATFULL on STN
1999:65064 Transdermal delivery system for antigen.
Alving, Carl R., Bethesda, MD, United States
Glenn, Gregory M., Bethesda, MD, United States

The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 5910306 19990608
APPLICATION: US 1996-749164 19961114 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 11 OF 18 USPATFULL on STN
1999:30374 Immunostimulating and immunopotentiating reconstituted influenza virosomes and vaccines containing them.
Gluck, Reinhard, Spiegel/Bern, Switzerland
Mischler, Robert, Worblaufen, Switzerland
Schweiz, Serum- & Impfinstitut Bern, Bern, Switzerland (non-U.S. corporation)
US 5879685 19990309
APPLICATION: US 1994-225740 19940411 (8)
PRIORITY: EP 1991-107527 19910508
EP 1991-107647 19910510
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 12 OF 18 USPATFULL on STN
1998:134594 Cationic lipids and the use thereof.
Unger, Evan C., Tucson, AZ, United States
Shen, Dekang, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5830430 19981103
APPLICATION: US 1995-391938 19950221 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 13 OF 18 USPATFULL on STN
1998:72264 Therapeutic drug delivery systems.
Unger, Evan C., Tucson, AZ, United States
Fritz, Thomas A., Tucson, AZ, United States
Matsunaga, Terry, Tucson, AZ, United States
Ramaswami, VaradaRajan, Tucson, AZ, United States
Yellowhair, David, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5770222 19980623
APPLICATION: US 1995-472305 19950607 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 14 OF 18 USPATFULL on STN
97:65876 Biologic bioadhesive compositions containing fibrin glue and **liposomes**, methods of preparation and use.
Marx, Gerard, New York, NY, United States
New York Blood Center, Inc., New York, NY, United States (U.S. corporation)
US 5651982 19970729
APPLICATION: US 1995-465888 19950606 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 15 OF 18 USPATFULL on STN
97:1183 Anti-viral therapeutic composition.
Tepic, Slobodan, AO-Forschungsinstitit AISF Research Institute Clavadelerstrasse, CH-7270 Davos, Switzerland
US 5591448 19970107
APPLICATION: US 1994-345465 19941121 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 16 OF 18 USPATFULL on STN

liposome dispersion.
Moynihan, Karen L., Brea, CA, United States
NeXstar Pharmaceuticals, Inc., Boulder, CO, United States (U.S. corporation)
US 5589189 19961231
APPLICATION: US 1994-306036 19940914 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 17 OF 18 USPATFULL on STN
94:9574 Methods of treating cancer using modified C-reactive protein.
Potempa, Lawrence A., Deerfield, IL, United States
Kresl, John J., Evanston, IL, United States
Anderson, Byron E., Morton Grove, IL, United States
Immtech International, Inc., Evanston, IL, United States (U.S. corporation)Northwestern University, Evanston, IL, United States (U.S. corporation)
US 5283238 19940201
APPLICATION: US 1992-874263 19920424 (7)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L13 ANSWER 18 OF 18 USPATFULL on STN
92:25125 Steroidal **liposomes** exhibiting enhanced stability.
Bolcsak, Lois E., Lawrenceville, NJ, United States
Boni, Lawrence, Monmouth Junction, NJ, United States
Popescu, Mircea C., Plainsboro, NJ, United States
Tremblay, Paul A., Hamilton, NJ, United States
The Liposome Company, Inc., Princeton, NJ, United States (U.S. corporation)
US 5100662 19920331
APPLICATION: US 1989-422047 19891016 (7)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d his

(FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004)

FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004
L1 33450 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L2 3069 S L1 AND (ALUMINUM HYDROXIDE)
L3 1785 S L1 AND (UNILAMELLAR)
L4 1772 S L3 AND LIPOSOME?
L5 434 S L4 AND (PHOSPHATIDYLCHOLINE)
L6 402 S L5 AND CHOLESTEROL
L7 107 S L6 AND MUCOSAL
L8 83 S L7 AND ORAL
L9 22 S L8 AND AY<2000
L10 13577 S L1 AND LIPOSOME?
L11 897 S L10 AND LIPOSOME?/CLM
L12 244 S L11 AND UNILAMELLAR
L13 18 S L12 AND UNILAMELLAR/CLM

=> s l13 and (cholesterol)
36219 CHOLESTEROL
L14 17 L13 AND (CHOLESTEROL)

=> s l14 and (phosphatidylcholine or phosphatidyl choline or PC)
8350 PHOSPHATIDYLCHOLINE
6952 PHOSPHATIDYL
18412 CHOLINE
3334 PHOSPHATIDYL CHOLINE
(PHOSPHATIDYL(W)CHOLINE)
116449 PC
L15 17 L14 AND (PHOSPHATIDYLCHOLINE OR PHOSPHATIDYL CHOLINE OR PC)

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=> s l15 and immunogenic/clm
          3327 IMMUNOGENIC/CLM
L16          2 L15 AND IMMUNOGENIC/CLM

=> d l16,cbib,1-
YOU HAVE REQUESTED DATA FROM 2 ANSWERS - CONTINUE? Y/(N):1-2
YOU HAVE REQUESTED DATA FROM 2 ANSWERS - CONTINUE? Y/(N):d
YOU HAVE REQUESTED DATA FROM 2 ANSWERS - CONTINUE? Y/(N):y

L16  ANSWER 1 OF 2  USPATFULL on STN
2004:57947 Liposomes.
    Gregoriadis, Gregory, London, UNITED KINGDOM
    US 2004043954 A1 20040304
    APPLICATION: US 2003-617734  A1  20030714 (10)
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L16  ANSWER 2 OF 2  USPATFULL on STN
2001:233138 Vaccines.
    Friede, Martin, Cardiff, CA, United States
    Garcon, Nathalie, Rixensart, Belgium
    SmithKline Beecham Biologicals s.a. (U.S. corporation)
    US 2001053365 A1 20011220
    APPLICATION: US 2001-819464  A1  20010328 (9)
    PRIORITY: GB 1995-8326  19950425
    GB 1996-910019  19960401
    GB 1996-20795  19961005
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.


=> d l16,cbib,ab,clm,kwic,2

L16  ANSWER 2 OF 2  USPATFULL on STN
2001:233138 Vaccines.
    Friede, Martin, Cardiff, CA, United States
    Garcon, Nathalie, Rixensart, Belgium
    SmithKline Beecham Biologicals s.a. (U.S. corporation)
    US 2001053365 A1 20011220
    APPLICATION: US 2001-819464  A1  20010328 (9)
    PRIORITY: GB 1995-8326  19950425
    GB 1996-910019  19960401
    GB 1996-20795  19961005
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB      The invention relates to a vaccine composition comprising an antigen, an
        immunologically active saponin fraction and a sterol.
CLM     What is claimed is:
        1. An adjuvant composition comprising an immunostimulatory saponin
        fraction derived from the bark of Quillaja Saponaria Molina as a single
        HPLC peak and a sterol, with the proviso that when the adjuvant
        formulation comprises an ISCOM the saponin is Qs21:

        2. An adjuvant composition as claimed in claim 1 wherein the
        immunologically active saponin fraction is derived from the bark of
        Quillaja Saponaria Molina is at least 90% pure.

        3. An adjuvant composition as claimed in any one of claim 1, wherein the
        immunologically active saponin fraction derived from the bark of
        Quillaja Saponaria Molina is QS21.

        4. An adjuvant composition as claimed in claim 1 wherein the sterol is
        in excess weight for weight to the immunologically active saponin
        fraction.
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5. An adjuvant composition as claimed in any one of claim 1 wherein the ratio of saponin:sterol is from 1:100 to 1:1 (w/w).

6. An adjuvant composition as claimed in claim 5 wherein the ratio of saponin:sterol is at least 1:2 (w/w).

7. An adjuvant composition as claimed in claim 6, wherein the ratio of saponin:sterol is 1:5 (w/w).

8. An adjuvant composition as claimed in claim 1, wherein the immunologically active saponin fraction derived from the bark of Quillaja Saponaria Molina is QS17.

9. An adjuvant composition as claimed in claim 1, wherein the sterol is **cholesterol**.

10. An adjuvant composition as claimed in claim 1, wherein the adjuvant composition is in the form of a vesicle.

11. An adjuvant composition as claimed in claim 10, wherein the adjuvant composition is in the form of a **liposome**.

12. An adjuvant composition as claimed in claim 11, wherein the adjuvant composition is in the form of a small **unilamellar liposome**.

13. An adjuvant composition as claimed in claim 10, wherein the adjuvant composition further comprises a phospholipid.

14. An adjuvant composition as claimed in claim 13, wherein the phospholipid is dioleoyl **phosphatidylcholine**.

15. An adjuvant composition comprising a saponin, a sterol, and a derivative of LPS.

16. An adjuvant composition as claimed in claim 15, wherein the LPS derivative is present in a lipid bilayer membrane.

17. An adjuvant composition as claimed in claim 15, wherein the derivative of LPS is a purified or synthetic lipid A of the following formula: ##STR4## wherein R2 may be H or PO3H2; R3 may be an acyl chain or β-hydroxymyristoyl or a 3-acyloxyacyl residue having the formula: ##STR5##

18. An adjuvant composition as claimed in claim 17, wherein the LPS derivative is 3-O-deacylated monophosphoryl lipid A.

19. An adjuvant composition comprising QS21, 3D-MPL and **cholesterol**.

20. An adjuvant formulation comprising a purified and stable QS21 saponin which is substantially devoid of hydrolysed QS21

21. An adjuvant formulation comprising 3D-MPL and a **liposome**, wherein the 3D-MPL is present in the lipid bilayer membrane.

22. An adjuvant composition as claimed in any one of claims 1 to 21, wherein the composition further comprises a carrier.

23. An adjuvant composition as claimed in claim 22, wherein the carrier is an oil in water emulsion or a metallic salt particle.

24. An adjuvant composition comprising a saponin, a sterol and a metallic salt particle.

25. An adjuvant composition as claimed in claim 24, wherein the metallic salt particle is aluminium hydroxide or aluminium phosphate.

is QS21.

27. An **immunogenic** composition comprising an adjuvant composition as claimed in any one of claims 1 to 21, further comprising an antigen or antigenic composition.

28. An **immunogenic** composition comprising an adjuvant composition as claimed in claim 22, further comprising an antigen or antigenic composition.

29. A vaccine composition as claimed in any one of claims 1 to 21, further comprising an antigen or antigenic composition.

30. A vaccine composition as claimed in claim 22, further comprising an antigen or antigenic composition.

31. A vaccine as claimed in claim 29, wherein the antigen is derived from any of **Human Immunodeficiency Virus**, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

32. A vaccine as claimed in claim 30, wherein the antigen is derived from any of **Human Immunodeficiency Virus**, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

33. A vaccine as claimed in claim 29 wherein the antigen is a tumour antigen.

34. A vaccine as claimed in claim 30 wherein the antigen is a tumour antigen.

35. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 27.

36. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 28.

37. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 29.

38. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition as claimed in claim 30.

39. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 27.

40. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 28.

41. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 29.

42. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition as claimed in claim 30.

43. A process for making a vaccine composition as claimed in claim 29, comprising admixing an immunologically active saponin fraction and **cholesterol** with an antigen or antigenic composition.

44. A process for making a vaccine composition as claimed in claim 30, comprising admixing an immunologically active saponin fraction and **cholesterol** with an antigen or antigenic composition.

45. A method of inducing CTL responses in a mammal comprising administering a vaccine composition as claimed in claim 29.

46. A method of inducing CTL responses in a mammal comprising administering a vaccine composition as claimed in claim 30.

47. A method of reducing the reactogenicity of QS21 containing adjuvant formulations, by the addition of excess sterol to the adjuvant formulation (weight/weight).

48. A method of stabilising QS21 against alkali mediated hydrolysis in QS21 containing adjuvant formulations, by the addition of excess sterol to the adjuvant formulation (weight/weight).

49. A process for the manufacture of an adjuvant formulation comprising making small **unilamellar liposomes** (SUV) comprising a sterol such as **cholesterol**, followed by the admixture of a saponin.

SUMM . . . and marine animal kingdoms. Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating **cholesterol**. When saponins are near cell membranes they create pore-like structures in the membrane which cause the membrane to burst. Haemolysis. . .

SUMM . . . also been disclosed as an adjuvant by Scott et al, Int. Archs. Allergy Appl. Immun., 1985, 77, 409. QuilA and **cholesterol** containing **liposomes** are described in Lipford et al., 1994, Vaccine, 12, 1, 73-80. Quil A immunogenic compositions are also described in Bomford,. . .

SUMM . . . Molina, and more particularly those which are isolated as an HPLC peak, such as QS21, and the preferred sterol is **cholesterol**. The adjuvants of the present invention may be in a particulate form, and may be formulated with a carrier, and. . .

DRWD [0008] FIG. 1 is a graph showing a comparison of QS21 quenching by **liposomes** containing or lacking **cholesterol**.

DRWD [0011] FIG. 4 is a graph showing anti-gp120 CTL activity generated by QS21 and **cholesterol** containing lipsome as adjuvant.

DETD [0020] Preferred sterols for use in the adjuvant compositions of the present invention include β-sitosterol, stigmasterol, ergosterol, ergocalciferol and **cholesterol**. These sterols are well known in the art, for example **cholesterol** is disclosed in the Merck Index, 11th Edn., page 341, as a naturally occurring sterol found in animal fat. Most preferably the sterol is **cholesterol**.

DETD . . . Other immunologically active saponin fractions useful in compositions of the invention include QA17/QS17. Compositions of the invention comprising QS21 and **cholesterol** show decreased reactogenicity when compared to compositions in which the **cholesterol** is absent, while the adjuvant effect is maintained. In addition it is known that pure QS21 degrades under basic conditions. . . advantage of the present compositions is that the stability of pure QS21 to base-mediated hydrolysis is enhanced in formulations containing **cholesterol**. Accordingly, there is provided an adjuvant formulation comprising a purified and stable QS21 saponin which is substantially devoid of hydrolysed. . .

DETD . . . Saponin and the sterol in a vesicle like structure. In particular, preferred adjuvants of the present invention are those forming a **liposome**. Compositions where the sterol/immunologically active saponin fraction forms an ISCOM structure also form an aspect of the invention, when the. . .

DETD . . . of the present invention the adjuvant formulation preferably further comprises a lipid capable of forming a bilayer membrane. Accordingly, the **liposomes** or ISCOMs preferably contain a neutral lipid, for example **phosphatidylcholine**, which is preferably non-crystalline at room temperature, for example eggyolk **phosphatidylcholine**, dioleoyl **phosphatidylcholine** or dilauryl **phosphatidylcholine**, and of these lipids dioleoyl **phosphatidylcholine** is most preferred. The vesicles may also contain a charged lipid which increases the stability of the **liposome**-QS21 structure for **liposomes** composed of saturated lipids. In these cases the amount of charged lipid is preferably 1-20% w/w, most preferably 5-10%. The ratio of sterol to phospholipid is 1-50% (mol/mol), most preferably 20-25%. Typically, if both are present, the sterol (**cholesterol**): **phosphatidylcholine** ratio is (1:4 w/w).

DETD [0027] The vesicular adjuvants of the present invention may be **unilamellar** or multilamellar. Most preferably the vesicles are **unilamellar liposomes**. The size of the vesicles are typically in the range of 10-1000 nm (mean particle size) and more preferably between 10-220 nm, and most preferably between 10-150 nm in size such as around 115 nm. Accordingly small **unilamellar** vesicles (SUV) with a mean diameter particle size of between 70-150 nm comprising the saponin and the sterol (preferably QS21 and **cholesterol**) where there is excess sterol present are particularly preferred adjuvants of the present invention.

DETD . . . be 5:1 weight to weight (w/w). For these vesicular embodiments of the present invention QS21 is the preferred saponin and **cholesterol** is the preferred sterol, and the above ratios apply to these molecules accordingly.

DETD . . . of Quillaja saponaria bark), a sterol, and a derivative of LPS. The most preferred adjuvant combination is QS21, 3D-MPL and **cholesterol**.

DETD . . . may be simply admixed with the saponin containing structures. Suitable compositions of the invention are those wherein the sterol/saponin containing **liposomes** or ISCOMs are initially prepared without the LPS derivative, and the LPS derivative is then added, preferably as particles with. . . also form an aspect of the invention. In this regard the adjuvant formulations preferably comprise a sterol and saponin containing **liposome**, and the LPS derivative (preferably 3D-MPL) is contained within the **liposome** membrane. Adjuvant formulations comprising 3D-MPL in the membrane of a liposomal formulation are particularly potent in the induction of cell. . .

DETD . . . salt particle, or be presented in an oil in water emulsion, or other suitable vehicle, such as for example, additional **liposomes**, microspheres or encapsulated antigen particles.

DETD [0041] Incorporation of aluminium salts in vaccine formulations containing an LPS derivative, a saponin fraction (such as QS21) and **cholesterol** containing SUV enhances both humoral and cellular responses and that vaccine formulations containing 3D-MPL, QS21, SUV and alum are non-toxic. . . In addition, the combined adjuvant appears to favour TH1 responses. In this regard, a preferred adjuvant formulation comprises QS21 and **cholesterol** containing SUV, adsorbed onto an aluminium salts, such as aluminium hydroxide or aluminium phosphate. A further enhancement of this adjuvant. . .

DETD . . . The adjuvants of the present invention may be manufactured using techniques known in the art. For example, the saponin and **cholesterol** may be admixed in a suitable detergent, followed by a solvent extraction technique to form the **liposomes** or ISCOMs of the present invention.

DETD . . . over the known methods. The preferred process by which the adjuvants of the present invention involves the manufacture of small **unilamellar liposomes** (SUV) comprising a sterol such as **cholesterol**, to which the saponin is admixed. For example a sample of

cholesterol-containing SUV (cSUV), may be added to QS21 at a ratio of 5:1 (**cholesterol**:QS21 w/w), which results in the QS21 associating with the liposomal bilayer membrane, which results in the formation of a liposomal structure. Alternatively, the cSUV may be added to the QS21 at a ratio of 1:5 (**cholesterol**:QS21 w/w), which results in the QS21 associating with the liposomal bilayer membrane and creating a "cage-like" ISCOM structure.

DETD [0044] In a preferred aspect of the invention, **liposomes**/SUV are first added to the QS21 and then mixed with alum which results in a significant proportion of the QS21 binding to the alum (via interaction through the **liposomes**). Such a formulation, when injected, is expected to result in a slower release of QS21 to the body, due to a depot effect of the alum, than if the QS21 was free or in un-fixed **liposomes**. The formulation containing 3D-MPL, QS21, SUV and alum are particularly advantageous as they are non-toxic and highly immunogenic.

DETD . . . the art can be used in the compositions of the invention, including polysaccharide antigens, antigen or antigenic compositions derived from **HIV**-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives. . .

DETD . . . New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Md., U.S.A. 1978. Encapsulation within **liposomes** is described, for example, by Fullerton, U.S. Pat. No. 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by. . .

DETD . . . adjuvant formulations, and also a method of stabilising QS21 against alkali mediated hydrolysis, by the addition of excess sterol (particularly **cholesterol**) to the adjuvant formulation (weight/weight).

DETD 1.1 Method of Preparation of **Liposomes**

DETD [0060] A mixture of lipid (such as **phosphatidylcholine** either from egg-yolk or synthetic) and **cholesterol** in organic solvent, is dried down under vacuum (or alternatively under a stream of inert gas). An aqueous solution (such. . . then added, and the vessel agitated until all the lipid is in suspension. This suspension is then microfluidised until the **liposome** size is reduced to 100 nm, and then sterile filtered through a 0.2 μm filter. Extrusion or sonication could replace. . .

DETD [0061] Typically the **cholesterol:phosphatidylcholine** ratio is 1:4 (w/w), and the aqueous solution is added to give a final **cholesterol** concentration of 5 to 50 mg/ml. If 3D-MPL in organic solution is added to the lipid in organic solution the final **liposomes** contain 3D-MPL in the membrane (referred to as 3D-MPL in).

DETD [0062] The **liposomes** have a defined size of 100 nm and are referred to as SUV (for small unilamelar vesicles). If this solution. . .

DETD [0063] The **liposomes** by themselves are stable over time and have no fusogenic capacity.

DETD [0064] QS21 in aqueous solution is added to the **liposomes**. This mixture is then added to the antigen solution which may if desired contain 3D-MPL in the form of 100. . .

DETD 1.3 The Lytic Activity of QS21 is Inhibited by **Liposomes** Containing **Cholesterol**

DETD [0065] When QS21 is added to erythrocytes, they lyse them releasing hemoglobin. This lytic activity can also be measured using **liposomes** which contain **cholesterol** in their membrane and an entrapped fluorescent dye, carboxyfluorescein--as the **liposomes** are lysed the dye is released which can be monitored by fluorescence spectroscopy. If the fluorescent **liposomes** do not contain **cholesterol** in their membrane no lysis of the **liposomes** is observed.

DETD [0066] If the QS21 is incubated with **liposomes** containing **cholesterol** prior to adding it to erythrocytes, the lysis of the erythrocytes is diminished depending on the ratio of **cholesterol** to QS21. If a 1:1 ratio is used no lytic activity is detected. If the **liposomes** do not contain **cholesterol**, inhibition of lysis requires a one thousand fold excess of phospholipid over QS21.

DETD [0067] The same holds true using fluorescent **liposomes** to measure the lytic activity. In FIG. 1, the lytic activity of 4 μg of QS21 treated

containing **cholesterol** (1 mg lecithin, 500 µg **cholesterol** per ml) was measured by fluorescence.

DETD [0068] The data shows that QS21 associates in a specific manner with **cholesterol** in a membrane, thus causing lysis (of cells or fluorescent **liposomes**). If the QS21 first associates with **cholesterol** in **liposomes** it is no longer lytic towards cells or other **liposomes**. This requires a minimum ratio of 0.5:1 chol:QS21 (w/w).

DETD [0069] **Cholesterol** is insoluble in aqueous solutions and does not form a stable suspension. In the presence of phospholipids the **cholesterol** resides within the phospholipid bilayer which can form a stable suspension of vesicles called **liposomes**. To avoid the requirement to add phospholipids a soluble derivative was tried. Polyoxyethanyl-**cholesterol** sebacate is soluble in water at 60 mg/ml however even at a 2000 fold excess (w/w) over QS21 no reduction. . .

DETD 1.4 Increased Stability of QS21 by **Liposomes** Containing **Cholesterol**

DETD . . . that at pH 9 and at a temperature of 37° C., 90% of QS21 is hydrolysed within 16 hours. If **liposomes** containing **cholesterol** are added to the QS21 at a ratio of 2:1 (chol:QS21 w/w) no hydrolysis of the QS21 is detected under. . .

DETD [0071] It is concluded that when QS21 associates with **liposomes** containing **cholesterol** it becomes much less susceptible to base-mediated hydrolysis. The hydrolysis product is described as having no adjuvant activity when given. . . QS21 have to be formulated at acidic pH and kept at 4° C. to maintain adjuvant composition. the use of **liposomes** may overcome this requirement.

DETD [0072] Mice injected in tibialis muscle with 5 µg QS21 (or digitonin) added to increasing quantities of **liposomes** (expressed in terms of µg **cholesterol**). Lytic activity is expressed as µg QS21 equivalent, which means that quantity of QS21 required to achieve the same hemolysis. . .

DETD [0073] The data shows that when the lytic activity is abolished by the addition of **liposomes** containing **cholesterol** the toxicity due to the QS21 is also abolished.

DETD [0075] The data shows that the addition of **cholesterol**-containing **liposomes** to the formulation significantly reduces the elevation in CPK (creatine phospho kinase) caused by the QS21. Since the CPK increase. . .

DETD 1.7 Binding of the **liposome**-QS21 complex to alum

DETD [0076] QS21 was incubated with neutral **liposomes** containing excess **cholesterol** as well as radioactive **cholesterol** and then incubated with alum ($Al(OH)_3$) in PBS. Alone, neither neutral **liposomes** nor QS21 bind to alum in PBS, yet negatively charged **liposomes** do. When together however, QS21 and neutral **liposomes** bind to alum. The supernatant contained neither QS21 (assayed by orcinol test) nor radioactive **cholesterol**.

DETD [0077] This indicates that the QS21 has bound to the **liposomes** and permits the **liposome**-QS21 combination to bind to the alum. This may arise from a negative charge being imposed on the **liposomes** by the QS21, or to an exposure of hydrophobic regions on the **liposomes**. The results also imply that QS21 does not extract **cholesterol** from the membrane.

DETD [0079] SUV were prepared by extrusion (EYPC:chol:3D-MPL 20:5:1). For MPL out, **liposomes** were prepared without 3D-MPL and 3D-MPL added as 100 nm particles.

DETD [0086] Additional results from a second experiment comparing QS21 and QS21 in the presence of **cholesterol** (SUV) in balb/c mice with HSV gD as antigen are shown below:

IgG 7 post IgG 14 post. . .

DETD [0087]

Liposomes = SUV containing MPL in the membrane
Chol:QS21 = 6:1

response was tested one weeks after the immunization.

IFN-g IL2 IL5

formulation. . .

DETD [0088] The data shows that QS21 associated with **cholesterol**-containing **liposomes** and MPL induces ThI/ThO response equal to MPL+QS21.

DETD [0089] At this ratio of **cholesterol** to QS21, QS21 is non-toxic in rabbits (by CPK).

DETD [0090] In a second experiment balb/c mice were immunised intra-footpad with gp120 in the presence of QS21 or QS21+SUV containing **cholesterol**. The cytotoxic T-lymphocyte activity in spleen cells was measured.

DETD [0091] This demonstrates that QS21 alone induces CTL activity, and that QS21 in the presence of **liposomes** containing **cholesterol** induces CTL activity at least as good as, or better than, QS21 alone.

DETD [0096] DQ QS21 (i.e. QS21/**cholesterol** or quenched QS21) may be tested in the woodchuck therapeutic model where animals are chronically infected with the virus. Specific. . .

DETD [0100] gD 5 μg+QS21 50 μg+SUV containing 50 μg **cholesterol**

DETD [0101] gD 5 μg+QS21 100 μg+SUV containing 100 μg **cholesterol**

DETD [0102] gD 5 μg+QS21 50 μg+SUV containing 250 μg **cholesterol**

DETD [0105] gD 5 μg+MPL 12.5 μg+QS21 12.5 g+SUV containing 62.5 μg **cholesterol**, or left untreated.

DETD [0117] The results are shown in the table below:

Prophylactic Model

Experiment 1 (chol refers to SUV containing **cholesterol**)

		PRIMARY DISEASE			
		Animal	Vaginal	External	PI
		without	lesions	lesions	Index**
	Lesion	lesion	incidence	incidence	reduction
n	FORMULATION	%	%. . .		severity*

DETD [0121] gD+MPL 50μg+QS21 50 μg+SUV containing 250 μg **cholesterol**,

DETD [0122] gD+Al(OH)3+MPL 50 μg+QS21 50 μg, +SUV containing 250 μg **cholesterol** or left untreated.

DETD [0125] A mixture of lipid (such as **phosphatidylcholine** either from egg-yolk or synthetic) and **cholesterol** in organic solvent, is dried down under vacuum (or alternatively under a stream of inert gas). An aqueous solution (such. . . then added, and the vessel agitated until all the lipid is in suspension. This suspension is then microfluidised until the **liposome** size is reduced to 100 nm, and then sterile filtered through a 0.2 μm filter. Extrusion or sonication could replace this step. Typically the **cholesterol**: phosphatidycholine ratio is 1:4 (w/w), and the aqueous solution is added to give a final **cholesterol** concentration of 5 to 50 mg/ml.

DETD [0127] QS21 (50 μg) is then added to SUV (containing between 50 and 250 μg **cholesterol**). This mixture is added to the above alum/antigen/MPL/buffer mixture. If required a bacteriostatic such as thiomersal is added (50 μg).

DETD [0128] The following Table shows the binding of QS21 to alum in the presence and absence of **liposomes** containing 25% (w/w) in dioleoyl **phosphatidylcholine**, and using a five-fold excess of **cholesterol** over QS21.

Formulation	SUV	μg QS21 bound
500 μg Alum + 50 μg QS21	0	<10
500 μg Alum + 50 μg. . .		

DETD [0129] In order to increase the binding of QS21 to alum, the quantity of

liposomes can be decreased. This decreases the cholesterol:QS21 ratio, however it has been shown that the QS21 remains non-toxic for **cholesterol**:QS21 ratios of 1:1 and greater. Table 2 shows that if the quantity of alum is decreased (from 500 µg to. . . of QS21 that is bound decreases significantly, and the quantity of MPL that is bound also decreases. By adding less **liposomes**, yet maintaining a **cholesterol**:QS21 ratio of 1:1 or greater, increased quantities of QS21 and MPL can be bound to the alum.

Formulation Chol/QS21 µg. . .

DETD . . . and comprises 283 amino acid from the mature N-terminal of the mature glycoprotein) with MPL and QS21 in combination with **liposomes** was tested with and without alum. The formulations were tested in African Green Monkeys.

DETD . . . immunised twice (0, 28 days) with 20 µg gD2t plus 50 µg MPL plus 50 µg QS21 with or without **liposomes** (250 µg **cholesterol** plus 1 mg DOPC) and with or without 500 µg alum. On day 42 the immune response was analysed.

DETD . . . was added. Then either SUV alone or a mixture of SUV and QS21 (50 µg QS21, SUV containing 250 µg **cholesterol** and 1 mg DOPC) were added. African green monkeys were injected three times with these formulations, or with FG on. . .

DETD . . . -SUV containing formulations in presence or not of Al(OH)3. MPL dose was 5µg, QS21 5 µg, SUV contained 25 µg **cholesterol** and 100 µg DOPC.

DETD . . . out) for 15 min. If needed, ten fold concentrated buffer is added before adding 5 ug of QS21 mixed with **liposomes** in a weight ratio QS21/**Cholesterol** of 1/5.

DETD Example 8, Production of QS2 and **Cholesterol** Containing **Liposomes**

DETD [0170] 40 mg of DOPC (dioleoyl **phosphatidylcholine**) and 10 mg of **cholesterol** was solubilised in choloroform and evaporated into a thin film by vaccuum dessication. The film was resuspended with 1 ml of PBS at pH 7.4 (10 mMPO4, 150 mM NaCl) to form multilamellar **liposomes** (MLV). The MLV were the microfluidised for 5 minutes (Microfluidiser M110S, which corresponds to 37.5 cycles), to form small **unilamellar** vesicles (SUV). 200 µl of SUV were then mixed with 200 µl of QS21 (stock of 1 mg/ml) which corresponds to a ratio of (5:1 **cholesterol**:QS21 w/w). The adjuvants were confirmed as **liposomes** (with the QS21 forming stable pores in the surface of the membrane) and not the cage-like ISCOM structure.

DETD . . . The same process is also used to insert 3D-MPL into the vesicle membrane, by adding 3D-MPL to the DOPC and **cholesterol** in the cholorform; and resuspending the film in PBS either at pH 7.4 or pH 6.1 (PBS 50 mMPO4 10. . .

DETD . . . liposomal adjuvant comprising the saponin QS21 and 3D-MPL (SUV-containing 50 µg 3D-MPLin the membrane, 50 µg QS21, and 250 µg **cholesterol** (chol:QS21 5:1 w/w)) in a total volume of 0.5 ml.

DETD . . . 50 µg 3D-MPL, 50 µg QS21, 50 µl oil in water emulsion (same as vaccine group 1) containing 100 µg **cholesterol** (chol:QS21 2:1 w/w) administered in a total volume of 0.5 ml.

DETD [0188] The adjuvants of the present invention, containing a saponin (QS21) and a sterol (**cholesterol**), induce high amounts of Hepatitis virus specific CTL in humans. The **cholesterol** containing adjuvants, together with the QS21, induced significantly better CTL responses that those QS21 containing adjuvants that did not contain **cholesterol**. Liposomal adjuvants (group2) of the present invention tended to induce higher amounts of CTL responses than other saponin (QS21) and sterol (**cholesterol**) containing formulations.

DETD [0189] In addition to the CTL responses, the **cholesterol** containing formulations were reported by the vaccinees to be less painful and induce fewer side effects of reduced severity compared to those reported for the non-**cholesterol** group 1. It is accepted in the scientific community that anti-HBs Ig titers superior or equal to 10 mIU/ml confer.

. .
9. An adjuvant composition as claimed in claim 1, wherein the sterol is **cholesterol**.

11. An adjuvant composition as claimed in claim 10, wherein the adjuvant composition is in the form of a **liposome**.

12. An adjuvant composition as claimed in claim 11, wherein the adjuvant composition is in the form of a small **unilamellar liposome**.

14. An adjuvant composition as claimed in claim 13, wherein the phospholipid is dioleoyl **phosphatidylcholine**.

19. An adjuvant composition comprising QS21, 3D-MPL and **cholesterol**.

21. An adjuvant formulation comprising 3D-MPL and a **liposome**, wherein the 3D-MPL is present in the lipid bilayer membrane.

27. An **immunogenic** composition comprising an adjuvant composition as claimed in any one of claims 1 to 21, further comprising an antigen or.
. .
28. An **immunogenic** composition comprising an adjuvant composition as claimed in claim 22, further comprising an antigen or antigenic composition.

31. A vaccine as claimed in claim 29, wherein the antigen is derived from any of **Human Immunodeficiency Virus**, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,. . .
32. A vaccine as claimed in claim 30, wherein the antigen is derived from any of **Human Immunodeficiency Virus**, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,. . .
. . . A process for making a vaccine composition as claimed in claim 29, comprising admixing an immunologically active saponin fraction and **cholesterol** with an antigen or antigenic composition.

. . . A process for making a vaccine composition as claimed in claim 30, comprising admixing an immunologically active saponin fraction and **cholesterol** with an antigen or antigenic composition.

49. A process for the manufacture of an adjuvant formulation comprising making small **unilamellar liposomes** (SUV) comprising a sterol such as **cholesterol**, followed by the admixture of a saponin.

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=> d his

     (FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004)

     FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004
L1         33450 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L2          3069 S L1 AND (ALUMINUM HYDROXIDE)
L3          1785 S L1 AND (UNILAMELLAR)
L4          1772 S L3 AND LIPOSOME?
L5           434 S L4 AND (PHOSPHATIDYLCHOLINE)
L6           402 S L5 AND CHOLESTEROL
L7           107 S L6 AND MUCOSAL
L8            83 S L7 AND ORAL
L9            22 S L8 AND AY<2000
L10        13577 S L1 AND LIPOSOME?
L11          897 S L10 AND LIPOSOME?/CLM
L12          244 S L11 AND UNILAMELLAR
L13           18 S L12 AND UNILAMELLAR/CLM
L14           17 S L13 AND (CHOLESTEROL)
```

L16 2 S L15 AND IMMUNOGENIC/CLM

=> s l15 not l16
L17 15 L15 NOT L16

=> d l17,cbib,1-15

L17 ANSWER 1 OF 15 USPATFULL on STN
2003:13075 Vaccine comprising an iscom consisting of sterol and saponin which is free of additional detergent.
Friede, Martin, Farnham, UNITED KINGDOM
Garcon, Nathalie, Wavre, BELGIUM
SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S. corporation)
US 6506386 B1 20030114
WO 2000007621 20000217
APPLICATION: US 2001-744800 20010604 (9)
WO 1999-EP5587 19990803
PRIORITY: GB 1998-17052 19980805
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 2 OF 15 USPATFULL on STN
2002:275943 Expression of cloned genes in the lung by aerosol and **liposome**-based delivery.
Debs, Robert James, Mill Valley, CA, United States
Zhu, Ning, San Francisco, CA, United States
The Regents of the University of California, Oakland, CA, United States (U.S. corporation)
US 6468798 B1 20021022
APPLICATION: US 1998-6841 19980114 (9)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 3 OF 15 USPATFULL on STN
2002:213456 Materials and methods for making improved **liposome** compositions.
Onyuksel, Hayat, Western Springs, IL, UNITED STATES
Rubinstein, Israel, Highland Park, IL, UNITED STATES
US 2002114829 A1 20020822
APPLICATION: US 2001-995263 A1 20011127 (9)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 4 OF 15 USPATFULL on STN
2002:136589 Archaesomes, archaeosomes containing coenzyme Q10 and other types of **liposomes** containing coenzyme Q10 adjuvants and as delivery vehicles.
Sprott, G. Dennis, Orleans, CANADA
Patel, Girishchandra B., Nepean, CANADA
Makabi-Panzu, Boby, Gatineau, CANADA
National Research Council of Canada, Ottawa, CANADA (non-U.S. corporation)
US 6403117 B1 20020611
APPLICATION: US 2000-612618 20000706 (9)
PRIORITY: US 1995-8724P 19951215 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 5 OF 15 USPATFULL on STN
2000:137879 Archaeosomes, archaeosomes containing coenzyme Q_{10}, and other types of **liposomes** containing coenzyme Q_{10} as adjuvants and as delivery vehicles.
Sprott, G. Dennis, Orleans, Canada
Patel, Girishchandra B., Nepean, Canada
Makabi-Panzu, Boby, Gatineau, Canada
Tolson, Douglas L., Victoria, Canada
National Research Council of Canada, Ottawa, Canada (non-U.S. corporation)

WO 9722333 19970626
APPLICATION: US 1998-77956 19980612 (9)
WO 1996-CA835 19961213 19980612 PCT 371 date 19980612 PCT 102(e) date
PRIORITY: US 1995-8724P 19951215 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 6 OF 15 USPATFULL on STN
2000:53722 Cationic lipids and the use thereof.
Unger, Evan C., Tucson, AZ, United States
Shen, Dekang, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
Imarx Pharaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 6056938 20000502
APPLICATION: US 1998-73181 19980505 (9)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 7 OF 15 USPATFULL on STN
1999:159514 Stabilized compositions of fluorinated amphiphiles for methods of therapeutic delivery.
Unger, Evan C., Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5997898 19991207
APPLICATION: US 1995-465868 19950606 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 8 OF 15 USPATFULL on STN
1999:65064 Transdermal delivery system for antigen.
Alving, Carl R., Bethesda, MD, United States
Glenn, Gregory M., Bethesda, MD, United States
The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 5910306 19990608
APPLICATION: US 1996-749164 19961114 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 9 OF 15 USPATFULL on STN
1999:30374 Immunostimulating and immunopotentiating reconstituted influenza virosomes and vaccines containing them.
Gluck, Reinhard, Spiegel/Bern, Switzerland
Mischler, Robert, Worblaufen, Switzerland
Schweiz, Serum- & Impfinstitut Bern, Bern, Switzerland (non-U.S. corporation)
US 5879685 19990309
APPLICATION: US 1994-225740 19940411 (8)
PRIORITY: EP 1991-107527 19910508
EP 1991-107647 19910510
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 10 OF 15 USPATFULL on STN
1998:134594 Cationic lipids and the use thereof.
Unger, Evan C., Tucson, AZ, United States
Shen, Dekang, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5830430 19981103
APPLICATION: US 1995-391938 19950221 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 11 OF 15 USPATFULL on STN

1996:72201 Therapeutic drug delivery systems.
Unger, Evan C., Tucson, AZ, United States
Fritz, Thomas A., Tucson, AZ, United States
Matsunaga, Terry, Tucson, AZ, United States
Ramaswami, VaradaRajan, Tucson, AZ, United States
Yellowhair, David, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5770222 19980623
APPLICATION: US 1995-472305 19950607 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 12 OF 15 USPATFULL on STN
97:65876 Biologic bioadhesive compositions containing fibrin glue and **liposomes**, methods of preparation and use.
Marx, Gerard, New York, NY, United States
New York Blood Center, Inc., New York, NY, United States (U.S. corporation)
US 5651982 19970729
APPLICATION: US 1995-465888 19950606 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 13 OF 15 USPATFULL on STN
96:120605 **Liposome** dispersion.
Moynihan, Karen L., Brea, CA, United States
NeXstar Pharmaceuticals, Inc., Boulder, CO, United States (U.S. corporation)
US 5589189 19961231
APPLICATION: US 1994-306036 19940914 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 14 OF 15 USPATFULL on STN
94:9574 Methods of treating cancer using modified C-reactive protein.
Potempa, Lawrence A., Deerfield, IL, United States
Kresl, John J., Evanston, IL, United States
Anderson, Byron E., Morton Grove, IL, United States
Immtech International, Inc., Evanston, IL, United States (U.S. corporation)Northwestern University, Evanston, IL, United States (U.S. corporation)
US 5283238 19940201
APPLICATION: US 1992-874263 19920424 (7)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L17 ANSWER 15 OF 15 USPATFULL on STN
92:25125 Steroidal **liposomes** exhibiting enhanced stability.
Bolcsak, Lois E., Lawrenceville, NJ, United States
Boni, Lawrence, Monmouth Junction, NJ, United States
Popescu, Mircea C., Plainsboro, NJ, United States
Tremblay, Paul A., Hamilton, NJ, United States
The Liposome Company, Inc., Princeton, NJ, United States (U.S. corporation)
US 5100662 19920331
APPLICATION: US 1989-422047 19891016 (7)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d l17,cbib,ab,kwic,1-15

L17 ANSWER 1 OF 15 USPATFULL on STN
2003:13075 Vaccine comprising an iscom consisting of sterol and saponin which is free of additional detergent.
Friede, Martin, Farnham, UNITED KINGDOM
Garcon, Nathalie, Wavre, BELGIUM

corporation)
US 6506386 B1 20030114
WO 2000007621 20000217
APPLICATION: US 2001-744800 20010604 (9)
WO 1999-EP5587 19990803
PRIORITY: GB 1998-17052 19980805
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The present invention provides an improved adjuvant formulation and a process for producing said adjuvant. The adjuvant comprises an ISCOM structure comprising a saponin, said ISCOM structure being devoid of additional detergent.
SUMM . . . the antigen was given alone are known in the art. These include metallic salts, such as aluminium hydroxide or phosphate; **liposomes**, the bacterially derived monophosphoryl lipid A, Cholera toxin, and numerous others. Adjuvants may be classed as immunostimulants which have a. . .
SUMM . . . are three dimensional `cage-like` structures which have been shown to form upon detergent removal from mixtures of saponins, detergents and **cholesterol**. ISCOMs and their use in vaccines are disclosed in EP 0 109 942 "Immunogenic protein or peptide complex, method of. . . higher sedimentation constant than the corresponding monomeric form of protein or peptide, and a higher sedimentation constant than the corresponding **liposome**. The classical "cage-like" structure of ISCOMs can be seen in the electron microscopy studies of EP 0 242 380 B1. . .
SUMM . . . stage, at a molar ratio of at least 0.1:1 of lipids to antigen, and preferably 1:1. Examples of lipids include **cholesterol** and **phosphatidyl choline**. Thus, a method for producing an immunogenic complex between an antigen and a polar triterpensaponin, associated by the attraction between. . .
SUMM 1. The formation of **cholesterol** containing small **unilamellar liposomes** (SUL) in the absence of detergent, and;
SUMM 2. Admixing the preformed **liposomes** with saponin at a ratio of saponin:**cholesterol** (w/w) exceeding 1.
SUMM . . . of any equipment, it is also susceptible to QC and GMP control throughout the process. For example a batch of **liposomes** may be produced and released for sterility and size, also a batch of saponin may be released for sterility and purity, all before the **liposomes** and saponin are admixed.
SUMM . . . invention may be produced and released from a QC point of view before the final step of admixing the pre-formed **liposomes** with the saponin. Additionally, the process of the present invention is not limited in the quantity of the final product.
SUMM . . . techniques of the art. Such processes which do not involve additional detergent include sonication, microfluidisation, or membrane extrusion. For example **phosphatidyl choline** (**PC**) dissolved in ethanol may be added to a flask and dried under vacuum or inert gas. PBS or other pharmaceutically. . . suspension may be microfluidised to attain a uniform preparation of SUL of around 100 nm in diameter. The SUL comprise **cholesterol** and also include one or more phospholipids. The ratio of **cholesterol** to phospholipid is at most 50% and preferably 20-25% (w/w). The phospholipid is preferably **phosphatidylcholine** and is most preferably chosen so as to have a low transition temperature e.g. Dioloeoylphosphatidylcholine or dilauryl **phosphatidylcholine**. Optionally a charged phospholipid (e.g. phosphatidylglycerol or phosphatidyl serine) may be added.
SUMM . . . between 1:1 to 100:1 (w/w), preferably between 1:1 to 10:1 (w/w), and most preferably 5:1 (w/w). The sterol is preferably **cholesterol**.
SUMM . . . antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from **HIV**-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein. . .

SUMM . . . applications EP 0 109 942, EP 0 180 564, and EP 0 242 380. In one preferred aspect the vaccine formulation of the invention comprises the **HIV**-1 antigen, gp120, especially when expressed in CHO cells. In a further embodiment, the vaccine formulation of the invention comprises gD2t. . .

SUMM . . . present invention is a process for the manufacture of an vaccine composition comprising the following steps: (1) the formation of **cholesterol** containing small **unilamellar liposomes** (SUL) in the absence of detergent; (2) admixing the preformed **liposomes** with saponin at a ratio of saponin:**cholesterol** (w/w) exceeding 1; (3) admixing an antigen with the product of step 2.

DETD Preparation of ISCOMS by Addition of QS21 to Small **Unilamellar Liposomes** (SUL).

DETD Dioloeoyl **phosphatidylcholine** (1 g) in ethanol was mixed with **cholesterol** (250 mg) in ethanol (solubilised by warming) and the ethanol removed under vacuum. Phosphate buffered saline (25 ml) was added and the flask agitated to suspend the lipids. The resulting multilamellar **liposomes** were microfluidised until the particle size was 100 nm as determined by photon correlation spectroscopy. The **liposomes** were filter sterilised through 0.22 μm filters and stored at 4° C.

DETD . . . 0.22 μm filters. To 100 μl of this solution (200 μg QS21) was added 20 μl SUL (containing 40 μg **cholesterol**).

DETD . . . ranging from 40 to 200 nm. FIG. 2 shows that when the QS21 is added at a five-fold excess over **cholesterol** the vesicular structures disappear and are replaced by spherical cage-like structures. These closely resemble the open spherical ISCOM structure. In. . .

DETD SUL were prepared as in example 1 except that a trace of radioactive (^{3H}) **cholesterol** was included and the SUL were prepared by sonication. ISCOMs were prepared by adding QS21 directly to these SUL at a QS21:**cholesterol** ratio of 5:1.

DETD . . . adding QS21 to the SUL was found further down the tube indicating the ISCOMs have a higher density than the **liposomes**.

CLM What is claimed is:

7. An-adjuvant composition as claimed in claim 1, wherein the sterol is **cholesterol**.

8. An adjuvant composition as claimed in claim 1, wherein the phospholipid is **phosphatidylcholine**.

9. An adjuvant composition as claimed in claim 8, wherein **phosphatidylcholine** is dioloeoylphosphatidylcholine or dilauryl **phosphatidylcholine**.

10. An adjuvant composition as claimed in claim 7, wherein the ratio of **cholesterol** to phospholipid is 50% (w/w).

11. An adjuvant composition as claimed in claim 10, wherein the ratio of **cholesterol** to phospholipid is 20-25% (w/w).

. . . vaccine composition as claimed in claim 12, wherein the antigen is an antigen or antigenic composition derived from any of **Human Immunodeficiency Virus**, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,. . .

14. A process for the manufacture of an adjuvant composition, comprising the following steps: (a) the formation of **cholesterol** containing small **unilamellar liposomes** (SUL) in the absence of detergent; and (b) admixing the preformed **liposomes** with saponin at a ratio of saponin:**cholesterol** (w/w) exceeding 1.

L17 ANSWER 2 OF 15 USPATFULL on STN

2002:275943 Expression of cloned genes in the lung by aerosol and **liposome**-based delivery.

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DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Methods and compositions for producing a mammal capable of expressing an exogenously supplied gene in cells of the airway are disclosed. **Liposome**-nucleic acid complexes are prepared then delivered via aerosol to the lung airway. The invention provides a direct method for transforming pulmonary cells, treat disorders of the lung, and for delivering substances systematically following expression in the lung.

TI Expression of cloned genes in the lung by aerosol and **liposome**-based delivery

AB . . . and compositions for producing a mammal capable of expressing an exogenously supplied gene in cells of the airway are disclosed. **Liposome**-nucleic acid complexes are prepared then delivered via aerosol to the lung airway. The invention provides a direct method for transforming. . .

SUMM . . . ex vivo techniques. Ex vivo techniques include transformation of cells in vitro with either naked DNA or DNA encapsulated in **liposomes**, followed by introduction into a host organ ("ex vivo" gene therapy). The criteria for a suitable organ include that the. . .

SUMM Retroviruses, adenoviruses and **liposomes** have been used in animal model studies in attempts to increase the efficiency of gene transfer; DNA has been introduced into animals by intratracheal (IT), intravenous, intraperitoneal, intramuscular, and intraarterial injection. Expression of introduced genes, either complexed to cationic **liposomes** or packaged in adenoviral vectors has been demonstrated in the lungs of rodents after IT instillation. However, IT injection is. . .

SUMM Hazinski, et al., Am. J. Respir. Cell Mol. Biol. (1991) 4:206-209, relates to **liposome**-mediated gene transfer of DNA into the intact rodent lung. Three fusion gene constructs were complexed to cationic **liposomes** including (1) the chloramphenicol acetyltransferase ("CAT") gene linked to a Rous sarcoma virus ("RSV") promoter; (2) the CAT gene linked to a mouse mammary tumor virus ("MMTV") promoter; and (3) a cytomegalovirus-β-galactosidase ("CMV-β-gal") fusion gene. The **liposome**/DNA complexes were instilled into the cervical trachea of rats and detectable levels of gene expression observed. Brigham et al, Am. J. Med. Sci. (1989) 298:278-281, describes the in vivo transfection of murine lungs with the CAT gene using a **liposome** vehicle. Transfection was accomplished by intravenous, intratracheal or intraperitoneal injection. Both intravenous and intratracheal administration resulted in the expression of. . . by the CMV promoter, in cultured bovine lung epithelial cells. The gene was added to cells in culture using cationic **liposomes**. The experimenters also detected the presence of α-1 antitrypsin in histological sections of the lung of New Zealand white rabbits following the intravenous delivery of gene constructs complexed to **liposomes**. Wolff et al., Science (1990) 247:1465-1468 relates to direct transfer of the CAT, β-gal and luciferase genes into mouse skeletal. . . observed in all three cases. Nabel et al., Science (1990) 249:1285-1288, pertains to in vivo intra-arterial transfection of pigs with **liposomes** containing a β-gal expression plasmid. Site-specific gene expression was observed in the arterial wall. None of the above cited art,. . .

SUMM . . . lung after intratracheal plasmid-mediated gene transfer. Debs et al. disclose pentamidine uptake in the lung by aerosolization and delivery in **liposomes**. Am Rev Respir Dis (1987) 135: 731-737.

SUMM . . . expresses an exogenously supplied gene of interest in cells of the lung. The method includes the steps of preparing a **liposome**-nucleic acid mixture suitable for nebulization, nebulizing the mixture, and depositing the resulting nebulized mixture in the lung of a mammalian. . .

DRWD FIG. 1 demonstrates that aerosol administration of pRSV-CAT-DOTMA: **cholesterol** complexes resulted in expression of the CAT gene in mouse

lungs. Lanes 1-3 were derived from mice receiving no treatment; lanes 4-6 represent mice administered 0.5 mg pRSV-CAT with 1.0 μmole DOTMA-**cholesterol liposomes**; lanes 7-9 were derived from mice receiving 2.0 mg pRSV-CAT alone; and lanes 10-12 represent mice given 2.0 mg pRSV-CAT with 4.0 μmol DOTMA-**cholesterol liposomes** in a 2 to 1 molar ratio. The CAT gene is not normally present in mammalian cells; the results thus indicate that the lung was successfully transfected by the pRSV-CAT DOTMA-**cholesterol**:**liposome** aerosol. The results also show that neither aerosol administration of the pRSV-CAT alone, nor a lower aerosol dose of pRSV-CAT: DOTMA-**cholesterol** complexes produce detectable expression of the CAT gene in mouse lungs. Thus, both the cationic **liposome** carrier, and a sufficient dose of DOTMA: **liposome** complexes are required to produce transgene expression in the lung after aerosol administration.

DRWD . . . the results of an experiment where mice were administered 12 mg of pCIS-CAT complexed to 24 μmoles of DOTMA/DOPE 1:1 **liposomes**. Lanes 1-3 show the results from animals administered the aerosol in an Intox-designed nose-only aerosol exposure chamber; lanes 4-7 are. . .

DETD . . . of phenotype of cells in the respiratory tract of a mammalian host following delivery of a sufficient dose of a **liposome**-nucleic acid aerosol to the host mammal. The **liposome**-nucleic acid aerosol is obtained by nebulization of a **liposome**-nucleic acid samplex mixture prepared in a biologically compatible fluid that minimizes aggregation of the **liposome**-nucleic complexes. The methods and compositions can be used to produce a mammal capable of expressing an exogenously supplied gene in. . .

DETD . . . to the lung via aerosol administration, and subsequently expressed in vivo. The instant invention takes advantage of the use of **liposomes** as a delivery mechanism. **Liposomes** are able to stably bind through charge interactions or entrap and retain nucleic acid and permit a system amenable to. . . can also be covalently conjugated to the liposomal surface so that nucleic acid can be delivered to specific cell types. **Liposomes** also allow for the delivery of relatively large amounts of nucleic acid, without a toxic effect, such that therapeutically effective amounts of the desired protein can be expressed in vivo. For a review of the use of **liposomes** as carriers for delivery of nucleic acids, see, Hug and Sleight, Biochim. Biophys. Acta. (1991) 1097:1-17; Straubinger et al., in. . .

DETD **Liposomes** have been used effectively, particularly to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcription factors and other cellular effectors into a variety of cultured cell lines and animals. In addition, successful clinical trials examining the effectiveness of **liposome**-mediated delivery of small drug molecules and peptides which act extracellularly have been reported. Several strategies have been devised to increase the effectiveness of **liposome**-mediated drug delivery by targeting **liposomes** to specific tissues and specific cell types. However, while the basic methodology for using **liposome**-mediated vectors is well developed and has been shown to be safe, the technique previously has not been perfected for **liposome**-based transfection vectors, and particularly not for aerosolized delivery to pulmonary tissue for in vivo gene therapy. By in vivo gene. . .

DETD In addition to discovering that transformation of lung cells can be obtained using aerosolized **liposome** nucleic acid constructs, Applicants have identified several factors that can affect the relative ability of particular **liposome**-nucleic acid constructs to provide transformation of lung cells following aerosolized delivery of a solution containing the **liposome**-nucleic acid constructs and to achieve a high level of expression. These factors include preparation of a solution that prior to. . . during nebulization will not form macroaggregates and wherein the nucleic acid is not sheared into fragments and preparation both of **liposomes** and of expression constructs, that provide for predictable transformation of host lung cells following aerosolization of the **liposome**-nucleic acid complex and administration to the host animal. These factors are discussed in detail below.

DETD Aerosol delivery of nucleic acid **liposome** complexes provides a number of advantages over other modes of administration. For example, aerosol administration can serve to reduce host. . . Montgomery et al., Chest (1989) 95:747-751; Leoung et al., N. Eng. J. Med. (1990) 323:769-775. Additionally, rapid clearance of circulating **liposomes** by the liver and spleen reticuloendothelial system is avoided, thereby allowing the sustained presence of the administered substance at the. . .

DETD Other advantages of the subject invention include ease of administration i.e., the host mammal simply inhales the aerosolized **liposome**-nucleic acid solution into the intended tissue, the lung. Further, by varying the size of the nebulized particles some control may. . .

DETD . . . on transgene insertion into retroviral or DNA virus vectors. Potential disadvantages of retrovirus vectors, as compared to the use of **liposomes**, include the limited ability of retroviruses to mediate in vivo (as opposed to ex vivo) transgene expression; the inability of. . .

DETD . . . further increase the incidence of transgene integration into a genomic DNA by incorporating a purified retroviral enzyme, such as the **HIV**-1 integrase enzyme, into the **liposome**-DNA complex. Appropriate flanking sequences are placed at the 5' and 3' ends of the transgene DNA. These flanking sequences have been shown to mediate integration of the **HIV**-1 DNA into host cell genomic DNA in the presence of **HIV**-1 integrase. Alternatively, the duration of the transgene expression in vivo can be prolonged by the use of constructs that contain. . .

DETD A particularly convenient method for obtaining nucleic acid for use in the **liposome**-nucleic acid preparations, is by recombinant means. Thus, the desired gene can be excised from a plasmid carrying the desired gene,. . .

DETD Preparation of **Liposomes**

DETD Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic **liposomes** are particularly preferred because a tight charge complex can be formed between the cationic **liposome** and the polyanionic nucleic acid, resulting in a **liposome**-nucleic acid complex which will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells after the aerosolized DNA:**liposome** complex has been deposited in the lung. Cationic **liposomes** have been shown to mediate intracellular delivery of plasmid DNA (Felgner, et al., Proc. Natl. Acad. Sci. USA (1987) 84:7413-7416);. . .

DETD **Liposomes** can be prepared from a variety of cationic lipids, including DOTAP, DOTMA, DDAB, L-PE, and the like. **Liposomes** containing a cationic lipid, such as {N(1-2-3-dioleyloxy) propyl}-N,N,N-triethylammonium} (DOTMA), dimethyl dioctadecyl ammonium bromide (DDAB), or 1, 2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) or lysinylphosphatidylethanolamine (L-PE) and a second lipid, such as distearoylphosphatidylethanolamine (DOPE) or **cholesterol** (Chol), are of particular interest. DOTMA synthesis is described in Felgner, et al., Proc. Nat. Acad. Sciences, (USA) (1987) 84:7413-7417. DOTAP synthesis is described in Stamatatos, et al., Biochemistry (1988) 27:3917. DOTMA:DOPE **liposomes** can be purchased from, for example, BRL. DOTAP:DOPE **liposomes** can be purchased from Boehringer Mannheim. **Cholesterol** and DDAB are commercially available from Sigma Corporation. DOPE is commercially available from Avanti Polar Lipids. DDAB:DOPE can be purchased. . .

DETD Cationic **liposome**:DNA complexes are internalized by cells by a classical receptor-mediated endocytosis using cell surface receptors which contain specific binding sites for,. . . the use of specific cationic lipids can confer specific advantages for in vivo delivery. For example, iv injection of DOTAP-containing **liposomes** can target transgene expression primarily to the lung and may offer increased advantages for aerosolized delivery. Furthermore, DOTAP, as well. . . DOTMA, are suitable for repeated administration to mammalian hosts. Additionally, complexing the cationic lipid with a second lipid, primarily either **cholesterol** or DOPE can maximize transgene expression in vivo. For example, mixing **cholesterol** instead of DOPE with DOTAP,

DETD Particular cells within the lung may be targeted by modifying the **liposomes** to direct them to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors may be. . . to target a cell associated with a particular surface protein. A particular ligand or antibody may be conjugated to the **liposome** in accordance with conventional ways, either by conjugating the site-directing molecule to a lipid for incorporation into the lipid bilayer. . .

DETD Non-cationic **liposomes**, particularly pH sensitive **liposomes**, offer another potentially attractive approach to in vivo gene therapy. However, as compared to cationic **liposomes**, pH sensitive **liposomes** are less efficient in capturing DNA and delivering DNA intracellularly. Anionic and neutral **liposomes** are readily available, such as from Avanti Polar Lipids (Birmingham, Ala.), or can be easily prepared using readily available materials. Such materials include **phosphatidylcholine**, **cholesterol**, phosphatidylethanolamine, dioleoylphosphatidylcholine (DOPC), dioleoylphoshatidylethanolamine(DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making **liposomes** using these materials are well known in the art.

DETD Unexpectedly, the liposomal lipid composition of the **liposomes** used for nebulization can dramatically affect the level of transgene expression produced in vivo. Thus, the liposomal lipid compositions generally. . . 50% molar ratio of cationic lipid to non-cationic lipid, but may range from 5% to 100%. The diameter of the **liposomes** should generally be within the range of 100 nm to 10 microns, preferably 100 nm to 500 nm. Other DNA sequences, such as adenovirus VA genes can be included in the **liposome**-DNA complex formulation or be co-transfected with the gene of interest. The presence of genes coding for the adenovirus VA gene. . .

DETD The **liposomes** can comprise multilamellar vesicles (MLVs), small **unilamellar** vesicles (SUVs), or large **unilamellar** vesicles (LUVs). MLV and LUV are prepared by vortexing rather than sonicating after addition of the aqueous material to the dry lipid film. If desired, the resulting **liposomes** can be extruded under high pressure through sized polycarbonate membranes to achieve more uniform size distributions. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of **unilamellar liposomes**. Commonly used methods for making **liposomes** include Ca^{2}+-EDTA chelation (Papahadjopoulos, et al., Biochim. Biophys. Acta (1975) 394:483; Wilson, et al., Cell (1979) 17:77); ether injection (Deamer,. . .

DETD The various **liposome**-nucleic acid complexes are prepared using methods well known in the art. See, e.g., Straubinger et al., in Methods of Immunology (1983), Vol. 101, pp. 512-527. By "**liposome**-nucleic acid complex" is meant a nucleic acid sequence as described above, either bound to the surface of, or entrapped in, a **liposome** preparation, as discussed below. The **liposome** preparation can also contain other substances, such as enzymes necessary for transcription and translation, cofactors, etc. Furthermore, the **liposome**-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types. MLVs containing nucleic acid. . . encapsulated and vortexing. The nucleic acid material is added to a suspension of preformed MLVs or SLVs only after the **liposomes** have been prepared and then vortexed. When using **liposomes** containing cationic lipids, the dried lipid film is resuspended in an appropriate solution such as sterile water or an isotonic buffer solution such as 10 mM Tris/NaCl, or 5% dextrose in sterile water, sonicated, and then the preformed **liposomes** are mixed directly with the DNA. The **liposome** and DNA form a very stable complex due to binding of the negatively charged DNA to the cationic **liposomes**. SUVs find use with small nucleic acid fragments as well as large regions of DNA (>250 kb).

DETD In preparing the **liposome**-nucleic acid complex, care should be taken to exclude any compounds from the solution which may promote the formation of aggregates of the **liposome**-nucleic acid complexes. Large particles generally will not be aerosolized by the nebulizer and even if

described would be too large to penetrate beyond the large airways. Aggregation of the **liposome**-nucleic acid complex is prevented by controlling the ratio of DNA to **liposome**, minimizing the overall concentration of DNA:**liposome** complex in solution, usually less than 5 mg DNA/8 ml solution, and avoiding chelating agents such as EDTA, and significant. . .

DETD The choice of **liposomes** and the concentration of **liposome**-nucleic acid complexes thus involves a two step process. The first step is to identify **liposomes** and concentration of **liposome**-nucleic acid complexes that do not aggregate when the components are combined or during the significant agitation of the mixture that. . .

DETD . . . reporter gene CAT (which encodes chloramphenicol acetyl transferase) can be inserted in the expression cassette and used to evaluate each **liposome** composition of interest. The DNA:**liposome** complexes must be mixed in solutions which do not themselves induce aggregation of the DNA:**liposome** complexes such as sterile water. The expression cassette (DNA) is mixed together with the **liposomes** to be tested in multiple different ratios, ranging as an example from 4:1 to 1:10 (micrograms DNA to nanomoles cationic lipid). The results will provide information concerning which ratios result in aggregation of the DNA:**liposome** complexes and are therefore not useful for use in vivo, and which complexes remain in a form suitable for aerosolization. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:**liposome** ratios confer the highest level of transgene expression in vivo. For example, the optimal DNA:**liposome** ratios for SUV for DOTMAIDOPE and DDAB:Chol are 1:1 or 1:2.

DETD . . . the invention, transformation in vivo is obtained by introducing a non-integrating therapeutic expression vector into the mammalian host complexed to **liposomes**, particularly cationic **liposomes**. For introduction into the mammalian host any physiologically acceptable medium may be employed for administering the DNA or **liposomes**, such as deionized water, 5% dextrose in water, and the like. Other components may be included in the formulation such. . .

DETD The **liposome**-nucleic acid complex is aerosolized by any appropriate method. For use with humans or other primates, the aerosol will be generated. . .

DETD . . . As an alternative to selecting small mean particle diameters to achieve substantial alveoli deposition, a very high dosage of the **liposome**-nucleic acid preparation can be administered, with a larger mean particle diameter. A proviso to such an approach is that the particular **liposome**-nucleic acid complex is not too irritating at the required dosage and that there are a sufficient number of particles in. . .

DETD . . . particle size generated thereby with e.g. a 7 stage Mercer cascade impactor (Intox Products, Albuquerque, N. Mex.). Concentrations of the **liposome**-nucleic acid complex from the impactor plates can be determined by eluting the complex therefrom and assessing the optical density at. . .

DETD The amount of **liposomes** used will be sufficient to provide for adequate transfection after entry of the DNA or complexes into the lung and. . . of expression to treat or palliate a disease of the host mammal following administration of an effective amount of the **liposome**-nucleic acid complex to the host mammal's lung, particularly the alveoli or airway. Thus, an "effective amount" of the aerosolized **liposome**-nucleic acid preparation, is a dose sufficient to effect treatment, that is, to cause alleviation or reduction of symptoms, to inhibit. . . reducing particular symptoms. Appropriate doses are discussed further below. While there is no direct method of measuring the amount of **liposome**-nucleic acid complex delivered to the alveoli, bronchoalveolar lavage (BAL) can be used to indirectly measure alveolar concentrations of the expressed. . .

DETD . . . type of nebulizer, the particle size, subject breathing patterns, severity of lung disease, concentration and the mean diameter of the **liposome**-nucleic acid complex in the aerosolized solution, and length of inhalation therapy. Thus, the amount of expressed protein

DETD . . . immune response to the treatment. The frequency of treatments depends upon a number of factors, such as the amount of **liposome**-nucleic acid complex administered per dose, as well as the health and history of the subject. As used herein, with reference to dosages, "**liposome**-nucleic acid aerosol" refers to the amount of **liposome**-nucleic acid complex that is placed in the nebulizer and subjected to aerosolization. The "amount nebulized" or "amount aerosolized" of the. . .

DETD . . . >70 mmHg), reduction in dyspnea, respiratory rate and/or fever. For the treatment of genetic disorders, such as cystic fibrosis, the **liposome**-nucleic acid complex will be administered at regular intervals, from once a week to once every one to several months, in. . .

DETD . . . for use in one or more procedures. Kits will usually include the DNA either as naked DNA or complexed to **liposomes**. Additionally, **liposomes** may be provided in a separate container for complexing with the provided DNA. The DNA or the **liposome**/DNA complexes may be present as concentrates which may be further diluted prior to use or they may be provided at. . . desired amount and concentration of agents. Thus, the kit may have a plurality of containers containing the DNA or the DNA/**liposome** complexes in appropriate proportional amounts. When the containers contain the formulation for direct use, usually there will be no need. . .

DETD Expression of chloramphenicol acetyltransferase (CAT) gene, in rodent lungs following aerosolized delivery of **liposome**-nucleic acid complexes.

DETD The pRSV-CAT plasmid was complexed to **liposomes** and administered to rodent subjects as follows. Two mg. of pRSV-CAT was mixed with 4 μmoles of DOTMA (GIBCO BRL, Grand Island, N.Y.)/**cholesterol** (2:1) small **unilamellar liposomes** in phosphate buffered saline and then nebulized in an Acorn I nebulizer (Marquest Medical Products, Inc., Inglewood, Colo.) to groups. . . only exposure chamber (intox Products, Albuquerque, N.M.). The same procedure was followed with 0.5 mg pRSV-CAT mixed with 1.0 μmol DOTMA-**cholesterol**, as well as 2.0 mg pRSV-CAT alone. Two to five days later, animals were sacrificed and lungs collected. Lungs were. . . et al., Science (1990) 247:1465-1468. As can be seen in FIG. 1, animals administered 2.0 mg RSV-CAT with 4.0 μmol DOTMA/**cholesterol** expressed the CAT protein while the control animals did not. A similar procedure was followed with respect to pRSV-β-gal, with the exception that 50 mg. of pRSV-β-gal was mixed with 50 μmoles of DOTMA/**cholesterol** (2:1). The presence of β-gal activity was determined using a standard histochemical staining procedure. β-gal activity was present in the. . .

DETD . . . placed in three different aerosol receiving chambers. All mice received the same amount of the CAT expression plasmid complexed to **liposomes**, as described above. Animals 1-3 were exposed to the aerosol in an Intox designed aerosol chamber. Animals 4-7 were exposed. . . assay. As can be seen in FIG. 2, a single aerosol dose of a CAT gene-expression plasmid complexed to cationic **liposomes** can produce high-level transgene expression in the lungs of mice. Significant levels of transgene expression are present in the lungs. . .

DETD Preparation of cationic **liposomes**

DETD **Liposomes** were prepared as small **unilamellar** vesicles (approximately 100 nm in diameter) containing the cationic lipid DOTMA as DOTMA:DOPE (1:1 mole ratio). DOTMA is (N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium(Syntex Corporation),. . . each, and the resulting mix was sonicated for approximately 20 minutes in a bath sonicator (Laboratory Supplies, Hicksville, N.Y.). The **liposomes** were stored under argon at 4° C. until use.

DETD Aerosol delivery of plasmid/**liposome** complexes to mice

DETD Twelve mg of plasmid complexed to 24 μmols of DOTMA:DOPE **liposomes** was aerosolized and administered to mice over two different aerosol periods on the same day. In order to prevent aggregation and precipitation of the oppositely charged components, the plasmid and the

liposomes were diluted separately in sterile water prior to mixing. Six mg of plasmid DNA and 12 μmols of DOTMA:DOPE **liposomes** were each diluted to 8 ml with water and mixed. Equal volumes were then placed into two Acorn I nebulizers. . .

DETD . . . or to an aerosol generated from a solution containing 12 mg of CMV-CAT complexed to 24 μmoles of DOTMA:DOPE (1:1) **liposomes**. Aerosols were administered to animals after they were placed individually in nose out cones and inserted into an Intox small. . . sacrificed 72 hours following aerosol administration demonstrated that significant CAT gene expression was seen only in mice exposed to aerosolized DNA/**liposome** complexes.

DETD . . . inter-animal variation, high levels of CAT activity are present for at least 21 days following a single aerosol dose of DNA/**liposome** complexes. No CAT activity was detectable in extracts from the heart, spleen, kidneys or liver of animals that showed high. . . expression in the lung, suggesting that the expression of the transgene was found to be lung-specific after aerosolization delivery of DNA-**liposome** complexes. This is consistent with prior observations showing that penetration of very high molecular weight substances through the respiratory epithelium of normal animals is very limited. Plasmid DNA/**liposome** complexes have molecular weights greater than 10^6.

DETD . . . lungs of mice within a single experiment were observed. One possible explanation for this variability is that the amount of DNA/**liposome** complex deposited in the lungs of mice is not uniform. In order to test this hypothesis, initial lung deposition of **liposomes** was measured using fluorescence analysis and of DNA was measured using Southern blot analysis. Either aerosolized cationic **liposomes** alone or DNA/**liposome** complexes containing 0.5 mole percent of a fluorescently labelled lipid, rhodamine-phosphatidylethanolamine were administered to mice.

DETD . . . experiment was actually deposited in the lung. In addition, there was no significant difference in lipid deposition between animals receiving **liposomes** alone and those receiving the DNA/**liposome** complexes. Since it is possible that a disruption of the complex could have occurred during nebulization, the amount of CAT gene deposited during aerosolization was also assessed. Immediately following aerosol delivery of DNA/**liposome** complexes, mice were sacrificed and total lung DNA prepared. Southern blots were probed with $\alpha[^{32P}]$-labelled CAT gene. Labelled bands were. . .

DETD . . . of lung cells which were transfected in vivo, lungs of mice sacrificed 72 hours following exposure to an aerosol containing DNA/**liposome** complexes were cryosectioned, probed with a polyclonal anti-CAT antibody and counterstained to detect intracellular CAT protein. Lung sections taken from DNA/**liposome** treated mice had a diffuse immunostaining pattern involving bronchiolar and alveolar components. The bronchiolar epithelial cytoplasm stained with greatest intensity. . .

DETD High level airway expression of the human CFTR gene in mouse lungs after aerosol administration of DDAB:**cholesterol liposome**-pZN32 complexes

DETD Preparation of cationic **liposomes**

DETD **Liposomes** were prepared as small **unilamellar** vesicles (approximately 100 nm in diameter) containing the cationic lipid DDAB (dimethyl dioctadecyl ammonium bromide) as DDAB **cholesterol** in a 1:1 molar ratio. DDAB was purchased from Sigma, St. Louis, Mo., and **cholesterol** was purchased from CalBioChem, San Diego, Calif. Stock solutions of the lipids were dissolved in chloroform. Lipids were mixed in. . .

DETD Aerosol delivery of plasmid/**liposome** complexes to mice

DETD Twelve mg of pZN32 complexed to 24 μmols of DDAB **cholesterol liposomes** was aerosolized over two different aerosol periods on the same day. To prevent aggregation and precipitation of the oppositely charged components, **liposomes** and DNA were diluted separately in sterile water prior to mixing. Six mg of plasmid DNA and 12 μmols of DDAB **cholesterol liposomes** were each diluted to 8 ml with water and mixed. Equal volumes of the DNA-**liposome** mixture were then placed into two Acorn I nebulizers (Marquest, Englewood, Colo.), and the animals

placed in an inlet small. . . 5 min was used to generate the aerosol. Ninety minutes were required to aerosolize this volume of 4 ml of DNA-**liposome** mixture. The animals were removed from the chamber for 1-2 hours and then the above procedure was repeated.

DETD Photomicrographs of frozen sections (viewed at different magnifications) of mouse lung 48 hours following aerosol exposure to pZN32-DDAB **cholesterol liposome** complexes and lung from untreated control showed intense staining with the polyclonal anti-CFTR antibody, α-1468, whereas the overwhelming majority of. . . airway cells are transfected with the human CFTR gene in vivo with a single aerosol dose of pZN32 complexed to DDAB-**cholesterol liposomes**. There was no histologic evidence of lung damage, inflammation or edema present in any of the pZN32-DDAB **cholesterol-liposome**-treated animals. pZN32-DDAB **cholesterol-liposome**-treated and control animals could not be distinguished histologically. The expression of the human CFTR gene is present in mouse lungs for at least 60 days following a single aerosol dose of pZN32 complexed to DDAB-**cholesterol liposomes**. Frozen sections of mouse lungs from control animals did not show any detectable staining for CFTR, confirming that all the. . .

DETD . . . by the above results, a single aerosol dose of an expression vector, containing a gene of interest, complexed to cationic **liposomes** transfects the majority of the cells lining both the conducting airways and the alveoli of the lung, the gene product. . . lung-specific, and there is no histological evidence of damage following exposure. There are several potential advantages to using aerosolized cationic **liposomes** as an in vivo gene delivery system. First, cationic **liposomes** can mediate efficient transfection of non-dividing cells. This is important because many airway epithelial cells are well differentiated and divide slowly or not at all. Second, **liposomes** (including **liposomes** containing cationic lipids) are non-infectious, and appear to be both well tolerated and non-immunogenic in a variety of human clinical trials. The effects of repeated aerosol administration of DNA/**liposome** complexes is effective and is non-toxic. More precise intrapulmonary targeting may be achieved by a) altering aerosol particle size to. . . preferentially direct the aerosol to alveoli or proximal versus distal airways or b) to covalently couple monoclonal antibodies to the **liposome** surface, thereby targeting lung cells expressing the corresponding cell surface antigen. Cationic **liposome**-mediated DNA delivery by aerosol provides high level, lung-specific transgene expression in vivo.

CLM What is claimed is:

1. A method of making an aerosolized lipid-nucleic acid complex composition, comprising: mixing a cationic **liposome** and a selected nucleic acid to form a cationic lipid-nucleic acid complex, which complex does not substantially aggregate in vitro;. . .

2. The method of claim 1, further comprising suspending the **liposome** and the nucleic acid, each in aqueous solutions, prior to mixing.

3. The method of claim 1, further comprising separately diluting the **liposome** or the nucleic acid prior to mixing in an aqueous buffer.

19. The method of claim 1, wherein the **liposome** and nucleic acid are mixed by agitation.

20. The method of claim 1, wherein the **liposome** is prepared by mixing constituent lipids of the **liposome** in chloroform, followed by evaporation of the chloroform and resuspension of the **liposome** in an aqueous solution.

21. The method of claim 1, wherein the **liposome** is prepared by drying a composition comprising the lipid components of the **liposome**, adding an aqueous solution to the resulting dried lipid components, and vortexing or sonicating the aqueous solution added to the dried lipid components to produce **liposomes**.

22. The method of claim 19, further comprising extruding the resulting

liposomes through a polycarbonate membrane.

23. The method of claim 1, wherein the cationic **liposome** comprises a non-cationic lipid.

24. The method of claim 1, wherein the cationic **liposome** comprises **cholesterol**.

25. The method of claim 1, wherein the cationic **liposome** is a small **unilamellar** vesicle (SUV).

26. The method of claim 1, wherein the cationic **liposome** is a multilamellar vesicle (MLV).

27. The method of claim 1, wherein the cationic **liposome** is a large **unilamellar** vesicle (LUV).

28. The method of claim 1, wherein the cationic **liposome** is mixed with the nucleic acid at a ratio of about 4:1 to about 1:10 micrograms nucleic acid to nanomoles. . .

29. The method of claim 1, wherein the cationic **liposome** is mixed with the nucleic acid at a ratio of about 1:1 to about 1:2 micrograms nucleic acid to nanomoles. . .

30. The method of claim 1, wherein the cationic **liposome** is between about 100 nm and 10 microns in diameter.

31. The method of claim 1, wherein the cationic **liposome** is between about 100 nm and 500 nm in diameter.

L17 ANSWER 3 OF 15 USPATFULL on STN

2002:213456 Materials and methods for making improved **liposome** compositions.

Onyuksel, Hayat, Western Springs, IL, UNITED STATES

Rubinstein, Israel, Highland Park, IL, UNITED STATES

US 2002114829 A1 20020822

APPLICATION: US 2001-995263 A1 20011127 (9)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Provided are methods for treating autism, multiple sclerosis, eneuresis, Parkinson's disease, amyotrophic lateral sclerosis, brain ischemia, stroke, Cerebral palsy sleep disorder, feeding disorder and AIDS-associated dementias, using improved biologically active **liposome** products comprising a biologically active amphipathic compound in association with a **liposome**. Methods for producing the **liposome** products as well as methods of using the **liposome** products in therapeutic and diagnostic techniques are also provided.

TI Materials and methods for making improved **liposome** compositions

AB . . . disease, amyotrophic lateral sclerosis, brain ischemia, stroke, Cerebral palsy sleep disorder, feeding disorder and AIDS-associated dementias, using improved biologically active **liposome** products comprising a biologically active amphipathic compound in association with a **liposome**. Methods for producing the **liposome** products as well as methods of using the **liposome** products in therapeutic and diagnostic techniques are also provided.

SUMM . . . presentation of amphipathic compounds, peptides, and proteins including analogs and fragments alone and/or conjugated to other compound in association with **liposomes** for both diagnostic and therapeutic uses.

SUMM . . . therapeutic methods for delivering peptides in the VIP/GRF or IL-2 family of peptides to targeted tissues through use of improved **liposome** compositions comprising a member of the VIP/GRF or IL-2 family of peptides and biologically active analogs, fragments and modulators thereof.

SUMM . . . injury. The peptide has been described to prevent neuronal cell death produced by the external envelope protein gp120 of the **human immunodeficiency virus** in vitro (Gozes et al., Mol. Neurobiol.

a potential therapy. . .

SUMM . . . of the peptide and thus enhance the biological actions of the peptide. Provision of the peptides expressed in and on **liposomes** would possibly permit intracellular delivery, since lipid bilayers of **liposomes** are known to fuse with the plasma membrane of cells and deliver entrapped contents into the intracellular compartment.

SUMM [0013] Characterization of the structure and properties of **liposomes** led to many proposed uses for the vesicle as vehicles to effect targeted drug delivery, most of which failed to materialize for any of a number of various reasons. Most prominently, the therapeutic parenteral use of conventional **liposomes** was found to be limited because of rapid uptake into the reticuloendothelial system by mononuclear phagocytic cells [Gregoriadias and Ryman,. . .

SUMM [0014] In attempts to overcome problems inherent to **liposome** drug delivery, research turned to several approaches including identification of compounds which would be released back into the blood following **liposome** uptake by the reticuloendothelial system, alternatives to intravenous **liposome** administration, and use of various compounds, for example, **cholesterol**, to increase **liposome** stability in the bloodstream [Kirby, et al., Biochem. J. 186-591-598 (1980); Hwang, in **Liposomes** from biophysics to therapeutics, Ostro (ed.) Marcel Decker: New York (1987) pp. 109-156; Beaumier, et al., Res. Comm. Chem. Pathol. Pharmacol. 39:227-232 (1983)]. Still other investigations examined various lipid compositions to form the **liposome** bilayer which more closely mimic the naturally occurring bilayer of red blood cell. Such efforts led to increased **liposome** half-life in circulation [Allen and Chonn, FEBS Lett. 223:42-46 (1987); Gabizon and Papahadjopoulos, Proc. Natl. Acad. Sci. (USA) 85:6949-6953 (1988)].

SUMM [0015] PCT Publication WO 95/27496 and Gao, et al., Life Science 54:247-252 (1994) describe the use of **liposomes** for delivery of VIP in comparison to its delivery in aqueous solution. Encapsulation of VIP in **liposomes** was found to protect the peptide from proteolytic degradation and to significantly enhance the ability of VIP and to effect a decrease in mean arterial pressure in comparison to VIP in aqueous solution in hypertensive hamsters. **Liposome**-associated VIP was found to significantly decrease mean arterial blood pressure for a period of approximately 12 minutes, with lowest blood pressure observed almost 5 minutes after initial administration. The publication also demonstrated binding of VIP in aqueous solution to **liposomes** and penetration of the peptide into the **liposome** bilayer. It was speculated that binding of VIP to **liposomes** might prevent loss of peptide activity either by partitioning of the peptide into the **liposome** membrane, stabilizing the peptide against proteolysis, or restricting the peptide in a biologically active conformation. Whatever the reason, encapsulation of VIP in **liposomes** enhanced in vivo biological activity of the peptide by both prolonging the effect and increasing the magnitude of the effect. . .

SUMM . . . et al., Proc. Intern. Symp. Control. Rel. Bioact. Mater. 17:77-78 (1990)]. This observation led to the development of sterically stabilized **liposomes** (SSL) (also known as "PEG-**liposomes**") as an improved drug delivery system which has significantly minimized the occurrence of rapid clearance of **liposomes** from circulation. [Lasic and Martin, Stealth **Liposomes**, CRC Press, Inc., Boca Raton, Fla. (1995)]. SSL are polymer-coated **liposomes**, wherein the polymer, preferably polyethylene glycol (PEG), is covalently conjugated to one of the phospholipids and provides a hydrophilic cloud. . . vesicle bilayer. This steric barrier delays the recognition by opsonins, allowing SSL to remain in circulation much longer than conventional **liposomes** [Lasic and Martin, Stealth **Liposomes**, CRC Press, Inc., Boca Raton, Fla. (1995); Woodle, et al., Biochem. Biophys. Acta 1105:193-200 (1992); Litzinger, et al., Biochem. Biophys.. . . and increases the pharmacological efficacy of encapsulated agents, as demonstrated for some chemotherapeutic and anti-infectious drugs [Lasic and Martin, Stealth **Liposomes**, CRC Press, Inc., Boca Raton, Fla. (1995)]. Studies in this area have demonstrated that different factors

of approximately 2,000 Da at a concentration of 5% (9-12) [Lasic and Martin, Stealth **Liposomes**, CRC Press, Inc., Boca Raton, Fla. (1995); Woodle, et al., Biochem. Biophys. Acta 1105:193-200 (1992); Litzinger, et al., Biochem. Biophys.. . . of SSL having desirable characteristics. This is particularly the case where an extrusion process is used to obtain small size **liposomes** with a narrow particle size distribution. For reasons which are not completely understood, such extrusion methods substantially reduce the biological activity peptide components associated with the **liposomes**. Accordingly, there remains a desire for improved **liposome** compositions which are sterically stable but which maintain the biological activity of associated peptide agents.

SUMM [0017] Also of interest to the present invention is the disclosure of PCT Publication WO 93/120802 which relates to multilamellar **liposomes** useful for enhancement of organ imaging with acoustics (ultrasound). The publication describes various **liposome** compositions ranging in size from 0.8 to 10 microns including a tissue specific ligand, such as an antibody, antibody fragment or a drug incorporated into the lipid bilayer, in order to facilitate tissue specific targeting. The oligolamellar **liposomes** are prepared by processes such as lyophilization, repeated freeze-thaw, or modified double emulsion techniques to produce internally separated bilayers. Preferred **liposomes** are said to range from 1.0 to 3.0 microns in diameter. It has thus far been more difficult to produce **liposomes** which are readily detectable by conventional ultrasound techniques less than about 0.5 microns in size. Accordingly, there remains a desire for improved **liposome** compositions which may be efficiently produced and which have average particle sizes less than about 0.5 microns. Moreover, there remains a desire for improved **liposome** compositions which are efficiently produced, stable in vivo, and provide a higher degree of resolution upon acoustic imaging.

SUMM [0018] Thus, there exists a need in the art to provide further improvements in the use of **liposome** technology for the therapeutic and diagnostic administration of bioactive molecules. More specifically, there remains a desire in the art for. . . improved methods for administration of amphipathic peptides including, but not limited to, members of the VIP/GRF family of peptides in **liposomes** in order to achieve a more prolonged and effective therapeutic effect.

SUMM [0019] The present invention provides methods of treating a variety of disease states using **liposome** compositions prepared as described in U.S. patent application Ser. No. 6, 197,333, issued Mar. 6, 2001, and PCT Publication No.. . . duration of the biological effect is believed to result, at least in part, from interaction of the compound with the **liposome** in such a manner that the compound attains, and is maintained in, an active or more active conformation than the. . . hormone releasing factor (GRF), hypocretins, pituitary adenylate cyclase activating peptide (PACAP), secretin, and glucagon. Preferred methods of the invention utilize **liposome** compositions comprising a member of the VIP/glucagon/secretin family of peptides including peptide fragments and analogs. The biologically active peptide products. . . it is desired to deliver a high level of biologically active compound or to , detect targeted delivery of the **liposome** product as will be described below.

SUMM . . . and AIDS-associated dementias, comprising the step of administering to an individual suffering from the disease state an amount of a **liposome** composition effective to alleviate conditions associated with the disease state, said **liposome** composition prepared by a method comprising the steps of: a) mixing a combination of lipids wherein said combination includes at least one lipid component covalently bonded to a water-soluble polymer; b) forming sterically stabilized **liposomes** from said combination of lipids; c) obtaining **liposomes** having an average diameter of less than about 300 nm; and d) incubating **liposomes** from step (c) with a biologically active amphipathic compound under conditions in which said compound becomes associated with said **liposomes** from step (c) in an active conformation, wherein at least one amphipathic compound is a member of the VIP/glucagon/secretin family of peptides including peptide fragments

and analogs. In one embodiment, methods of the invention employ active **liposome** compositions which comprise **unilamellar liposomes**. In another embodiment, these **liposome** compositions are multivesicular **liposomes**. In aspects of the invention wherein the **liposome** compositions are multivesicular **liposomes**, methods are provided wherein the **liposome** compositions produced by carrying out the steps of sequentially dehydrating and rehydrating **liposomes** obtained in step (c) with said biologically active peptide.

SUMM [0021] Preferably, methods utilize **liposome** compositions wherein the water soluble polymer is polyethylene glycol (PEG). Also preferred are methods wherein the amphipathic compound is characterized. . .

SUMM [0022] Methods of the invention include those wherein **liposomes** obtained in step (c) have an average diameter or less than about 200 nm. In a preferred aspect, the **liposomes** obtained in step (c) have an average diameter or less than about 100 nm. In one aspect, the **liposomes** are obtained in step (c) by extrusion to form **liposomes** having a selected average diameter. Alternatively, methods employ **liposome** which are obtained in step (c) by size selection.

SUMM [0023] In one aspect, methods of the invention utilize **liposome** which are formed from a combination of lipids that consists of distearoyl-phosphatidylethanolamine covalently bonded to PEG (PEG-DSPE), **phosphatidylcholine** (**PC**), and phosphatidylglycerol (PG) in further combination **cholesterol** (Chol). In a preferred method, these lipids are combined with **cholesterol** in a PEG-DSPE:**PC**:PG:Chol molar ratio of 0.5:5:1:3.5.

DETD [0024] The present invention provides improved methods of preparing biologically active **liposome** products comprising biologically active amphipathic compounds in association with a **liposome**. The preferred amphipathic compounds are characterized by having hydrophilic and hydrophobic domains segregated to the extent that the hydrophobic domain is capable of associating with or within the **liposome** bilayer. Compounds of the invention preferably attain a biologically active conformation in association with or within the **liposome** bilayer. Active conformations are those in which the desired compound is most likely to be capable of effecting its normal. . . the VIP/glucagon/secretin or IL-2 family of peptides including peptide fragments and analogs. While biologically active compounds are associated with the **liposome** bilayer, the association is not irreversible and the compound may be released either quickly or over time from association with the **liposome**, depending on properties of the **liposome** and the compound.

DETD [0025] In contrast to prior art methods which frequently include the step of extruding peptide-containing **liposomes** through membranes and filters to obtain **liposomes** of a desired size, the **liposomes** according to the present invention are obtained having a diameter of less than 300 nm prior to being contacted with the active compound ingredient. **Liposomes** of this size may be obtained using an extrusion step which modifies **liposomes**, thereby reducing the size of the **liposomes** to a preferred average diameter prior to being incubated with the biologically active compound. Alternatively, **liposomes** of the desired size may be selected using techniques such as filtration or other size selection techniques. While the size-selected **liposomes** of the invention should have an average diameter of less than about 300 nm, it is preferred that they are. . . about 200 nm with an average diameter of less than about 100 nm being particularly preferred. When the biologically active **liposome** product is a **unilamellar liposome**, it preferably is selected to have an average diameter of less than about 200 nm. The most preferred **unilamellar liposomes** of the invention have an average diameter of less than about 100 nm. It is understood, however, that multivesicular **liposomes** of the invention derived from smaller **unilamellar liposomes** will generally be larger and may have an average diameter of about less than 1000 nm. Preferred multivesicular **liposomes** of the invention have an average diameter of less than about 800 nm, and less than about 500 nm while most preferred multivesicular **liposomes** of the invention have an average diameter of less than about 300 nm.

DETD [0026] **liposomes** according to the invention may be produced from combinations of lipid materials well known and routinely utilized in the art to produce **liposomes** and including at least one lipid component covalently bonded to a water-soluble polymer. Lipids may include relatively rigid varieties, such. . . preferred polymer of the invention is PEG at a molecular weight between I 000 and 5000. Preferred lipids for producing **liposomes** according to the invention include distearoyl-phosphatidylethanolamine covalently bonded to PEG (PEG-DSPE), **phosphatidylcholine** (**PC**), and phosphatidylglycerol (PG) in further combination with **cholesterol** (Chol). According to a preferred embodiment of the invention, a combination of lipids and **cholesterol** for producing the **liposomes** of the invention comprise a PEG-DSPE:**PC**:PG:Chol molar ratio of 0.5:5:1:3.5.

DETD [0027] The **liposomes** produced according to the methods of the invention are characterized by improved stability and biological activity and are useful in. . . variety of therapeutic, diagnostic and/or cosmetic applications. According to one embodiment, the invention comprehends a composition comprising a biologically active **liposome** product wherein said biologically active amphipathic compound has anti-oxidant activity, anti-aging, anti-wrinkle formation or wound healing capacity. Compositions of this. . . for the treatment of a gastrointestinal disorder wherein said preparative method further comprises the step of encapsulating the biologically active **liposome** product in an enteric coating. The oral controlled release preparation is useful in a variety of gastrointestinal disorders including those. . . oral preparation includes a biologically active member of the VIP/glucagon/secretin or IL-2 family of peptides including peptide fragments and analogs. **Liposome** preparations comprising a biologically active member of the VIP/glucagon/secretin or IL-2 family of peptides including peptide fragments and analogs are. . . tissue, or cell type for storage and transplantation in a recipient comprising the step of incubating said organ in a **liposome** composition comprising a member of the VIP/glucagon/secretin or IL-2 family of peptides including peptide fragments and analogs.

DETD . . . methods of administering a biologically active amphipathic compound to a target tissue comprising the steps of: preparing a biologically active **liposome** product comprising a biologically active amphipathic compound in association with a **liposome** according to the methods of the invention and administering a therapeutically effective amount of the **liposome** product to said target tissue. The **liposome** products of the invention may be administered intravenously, intraarterially, intranasally such as by aerosol administration, nebulization, inhalation, or insufflation, intratracheally,. . .

DETD . . . than the biologically effective amount of VIP in aqueous solution. Regardless of which bioactive compound is associated with SSL, the **liposome** product must be tested in order to determine a biologically effective amount required to achieve the same result effected by. . .

DETD [0033] The invention further provides improved diagnostic compositions comprising multivesicular biologically active **liposome** products and methods for their use comprising the steps of: preparing a biologically active **liposome** product comprising a biologically active amphipathic compound in association with a multilamellar **liposome** prepared according to the methods of the invention; administering a diagnostically effective amount of the **liposome** product to a target tissue; and detecting uptake or interaction of the **liposome** product at the target tissue. According to one aspect of the invention, the target tissue is a tumor. In one aspect of the method, the **liposome** product is detectably labeled with a label selected from the group including a radioactive label, a fluorescent label, a non-fluorescent. . . a dye, or a compound which enhances magnetic resonance imaging (MRI). According to the preferred embodiment of the invention, the **liposome** product is detected by acoustic reflectivity. Diagnostic **liposome** products for detection by acoustic imaging generally have an average diameter of less than about 1000 nm, but preferably, the diagnostic **liposome** products have an average diameter of less than 600 nm and most preferably have an

average diameter of less than. . .

DETD [0034] The invention also provides use of a biologically active **liposome** product comprising a biologically active amphipathic compound and produced according to methods of the invention for the treatment of inflammation,. . .

DETD . . . U.S. Pat. No. 6,197,333, the disclosure of which is hereby incorporated described results described results of use of VIP associated **liposomes** according to the invention. Specifically, VIP-PEG-**liposomes** were prepared as follows. DSPE linked to PEG (molecular weight 1,900), PG, **PC**, and **cholesterol** (molar ration 0.5:1:5:3.5) were dissolved in chloroform in a round bottom flask. The solution was dried overnight in a rotoevaporator. . . The lipid film was rehydrated with saline, pH 6-7, while vortexing, and then sonicated for at least 5 minutes. The **liposome** preparation thus formed was extruded through stacked Nucleopore filters with pore sizes 200nm, 100 nm, and 50 nm, respectively, until the mean size of PEG-**liposome** was 80-100 nm as determined by quasi elastic light scattering. VIP and trehalose, a cryoprotectant, were added to the extruded **liposome** preparation in polypropylene tubes, the mixture snap-freezed in ethanol- or acetone-dry ice bath for at least 20 minutes, and lyophilized overnight under similar conditions. Free VIP was separated from VIP-PEG-**liposomes** using Bio Gel A-5m column chromatography. The size of the PEG-**liposomes** in original solution and VIP-PEG-**liposomes** was determined by quasi elastic light scattering. Lipid concentration in PEG-**liposomes** in the original solution and in VIP-PEG-**liposomes** was determined by inorganic phosphate assay. VIP concentration in VIP-PEG-**liposomes** was determined by an ELISA assay.

DETD [0036] To determine VIP concentration in VIP-PEG-**liposomes**, 1% sodium dodecyl sulfate, a detergent, was added to an aliquot of the VIP-PEG-**liposome** preparation to release associated VIP before assay. PEG-**liposome** and 1% sodium dodecyl sulfate alone did not interfere with the ELISA assay. Non-limiting examples from preliminary experiments using these. . .

DETD [0037] U.S. Pat. No. 6,197,333 further disclosed that a bolus intravenous injection of 1.0 nmol VIP-PEG-**liposome** compound acted to decrease mean arterial pressure (MAP) in hamsters with spontaneous hypertension. The results are reproduced herein as FIGS.. . . indicated a significant, gradual and sustained decrease in mean arterial pressure reaching a nadir within 2 hours after injection of VIP-PEG-**liposomes** which lasted throughout the observation period of 7 hours.

DETD [0038] According to another experiment, normotensive hamsters were suffused onto the cheek pouch for 7 minutes with 0.1 nmol VIP-PEG-**liposome** composition which produced a significant increase in mean arterial diameter in situ. The results of this experiment are shown in. . .

DETD [0039] In still another experiment, 1.0 nmol VIP-PEG-**liposome** composition was superfused for 30 minutes into the nostril of a hypertensive hamster which resulted in a decrease in arterial. . .

DETD [0040] Finally, as another experiment the effect of VIP-PEG-**liposomes** on neutrophil chemotaxis was examined using a two chamber apparatus routinely employed for in vitro analysis of chemotaxis. The results. . . VIP alone in the lower chamber was shown to be negligible, and minor levels of neutrophil migration were detected against VIP-PEG-**liposomes** and PEG-**liposome** in the lower chamber. When neutrophils and VIP were added together in the upper chamber, significant migration was observed against fmlp in the lower chamber, with slightly lower levels of cell migration observed against fmlp with neutrophils and PEG-**liposomes** together in the upper chamber. Finally, neutrophil migration against fmlp was reduced to almost negligible levels when VIP-PEG-**liposomes** were added with the cells in the upper chamber. These results indicated that VIP-PEG-**liposomes** were capable of chemotactic inhibition of neutrophil migration in response to fmlp.

DETD . . . is a comparative example describing the state of the art which illustrates that incorporation of a bioactive VIP peptide into **liposomes** increases the duration and magnitude of the peptide activity

which administered to hamsters with spontaneous hypertension. Example 2 relates to an examination of the same biologically active peptide in association with a sterically stabilized **liposome** (SSL) according to the methods of invention but in which the **liposome** provides an even more dramatic increase in peptide activity. Example 3 provides an alternative method for preparing an SSL according. . . are shown to result in vastly different levels of peptide activity. Example 4 provides an analysis of morphological features in **liposomes** prepared by the methods described in Example 3. Example 5 relates to a modified method for producing SSL with a. . . simplification of the preparative process does not affect peptide activity in vivo. Example 6 describes manufacture and use of diagnostic **liposome** products for use in acoustic reflective imaging based on echo-reflective properties of the **liposomes**. Example 7 relates to the ability of DSPE-PEG5000 to interact with and stabilize interleukin-2 (IL-2) in aqueous medium. Example 8. . .

DETD Bioactivity of Peptides in Conventional **Liposomes**

DETD [0042] According to this example, prior art methods for incorporation of VIP into **liposomes** were reproduced in order to provide a basis for comparison of the methods of the invention. Because previous observations have. . . of VIP could elicit vasodilation in peripheral microcirculation of hamsters with spontaneous hypertension and whether encapsulation of VIP into conventional **unilamellar liposomes** could modulate any observed response.

DETD [0046] **Liposomes** containing VIP were prepared according to the methods of Gao, et al., Life Sci. 64: PL274-PL252 (1994); Gregoriadis and Florence,. . . Biophys. Acta 1061:297-303 (1991); and Suzuki, et al., Life Sci. 57:1451-1457 (1995). Briefly, a lipid composition consisting of egg yolk **phosphatidylcholine** (Sigma, St. Louis, Mo.), egg yolk phosphatidylglycerol (Sigma), and **cholesterol** (Sigma) at a 4:1:5 molar ratio (total phospholipid content, 5 mg) was mixed in chloroform (Sigma) and the solvent evaporated. . . (pore size 3 μm; Nuclepore, Pleasanton, Calif.) using a LiposoFast apparatus (capacity of syringe, 0.5 ml; Avestin, Ottawa, ON, Canada). **Liposomes** were collected using a disposable gel filtration column (Econo-pac IODG, polyacrylamide gel, 10 ml bed vol.) in 0.15 N NaCl [MacDonald, et al., Biochim. Biophys. Acta 1061:297-303 (1991)]; the **liposome** fraction was recovered in the void volume and stored at 4° C. until use.

DETD [0048] VIP alone or encapsulated in **liposomes** was suffused for 7 minutes at a concentration of VIP of either 0.05 or 0.1 nmol peptide, and more than. . .

DETD [0050] With suffusion of VIP at the same amounts but encapsulated in **liposomes**, normotensive animals showed significant, concentration-dependent potentiation and prolongation of vasorelaxant effects in comparison with VIP alone. The maximal response was. . . minutes after suffusion began and significant vasodilation persisted almost 9 minutes after suffusion was stopped. In hamsters with spontaneous hypertension, **liposome** encapsulated VIP produced a significant vasorelaxant effect of magnitude similar to that observed in the normotensive animals. A maximal effect. . . start of suffusion and significant vasodilation persisted over 3 minutes after suffusion was stopped. Even though encapsulation of VIP in **liposomes** was able to restore vasorelaxant effects of the peptide in hamsters with spontaneous hypertension to a magnitude similar to that. . .

DETD in Sterically Stabilized **Liposomes**

DETD [0052] Having demonstrated that VIP encapsulation in conventional **liposomes** restored capacity of the peptide to induce vasodilation in hamsters with spontaneous hypertension, changes in VIP activity when associated with the sterically stabilized **liposomes** of the invention were examined.

DETD [0054] Sterically stabilized **liposomes** (SSL) were prepared as follows. Egg yolk **phosphatidylcholine** (Sigma), egg yolk phosphatidylglycerol (Sigma), **cholesterol** (Sigma) and polyethylene glycol (molecular mass, 1,900) linked to distearoyl-phosphatidylethanolamine (molar ratio, 5:1:3.5:0.5; phospholipid content, 17 mmol) were dissolved and mixed in chloroform [Gao, et al., Life Sci. 54: PL247-PL252 (1994); Lasic and

[illegible] **liposomes**, CRC Press, Inc.: Boca Raton, Fla., 1990; Suzuki, et al., Am. J. Physiol. 271:H282-H287 (1996)]. The solvent was evaporated at. . .

DETD a Function of **Liposome** Preparation

DETD [0062] Having demonstrated that VIP in SSL exhibits enhanced bioactivity over VIP preparations in conventional **liposomes**, alternative methods of preparation were examined in order to determine optimal compositions, methods of their preparation, and to further characterize. . .

DETD [0063] Two different methods of **liposome** preparation methods were utilized. In both, the lipids distearoyl-phophatidylethanolamine (PEG-DSPE) (Sequus Pharmaceuticals, Menlo Park, Calif.), Egg yolk **phosphatidylcholine** (**PC**) (Sigma Chemical Co., St. Louis, Mo.), and egg yolk phosphatidylglycerol (PG) (Sigma Chemical Co., St. Louis, Mo.), were combined with **cholesterol** (Sigma Chemical Co., St. Louis, Mo.) at a PEG-DSPE:**PC**:PG:Chol molar ratio of 0.5:5:1:3.5. Total phospholipid content of the mixture was 17 pmol. The mixture was mixed in chloroform in. . .

DETD . . . the invention), VIP was initially mixed with a lipid composition followed by extrusion and repeated freezing and thawing to produce **liposomes**. Briefly, the dry lipid film was rehydrated with 250 μl 0.15 M saline (0.9% w/w NaCl) containing 0.4 mg VIP. . . suspension was extruded through polycarbonate filters using the Liposofast apparatus (pore size 200 nm, AVESTIN, Inc., Ottawa, ON, Canada). The **liposome**-associated VIP was separated from the free VIP by column chromatography (BioGel A-5m, Bio-Rad Laboratories, Richmond, Calif.) and stored at 4°. . . al., J. Pharm. Sci. In press (1996)] with a Nicomp 270 particle sizer (Particle Sizing Systems, Santa Barbara, Calif.) and **liposomes** prepared by this method were found to have an average mean diameter of 224±36 nm.

DETD . . . which is contemplated by the invention, a lipid mixture was first extruded, after which VIP was mixed with the formed **liposomes**. Briefly, a dry lipid film prepared as before was rehydrated with 250 ml 0.15 M saline without VIP. The mixture. . . in close contact which provides a promotes passive drug loading. Column separation and storage conditions were the same as above. **Liposomes** prepared by this method were found to have an average diameter of 250±50 run by the method described above, suggesting that freeze-drying permitted vesicle fusion. VIP concentration in the **liposomes** was determined after treatment with sodium dodecyl sulfate 1% by a VIP ELISA assay kit (Peninsula Laboratories, Belmont, Calif.) and. . . Elsevier, N.Y. (1972) pp. 354-356]. For both methods of preparation, approximately 30% of the starting VIP was found to be **liposome** associated and approximately 50% of the starting phospholipids was recovered giving a ratio of approximately 0.004 mole VIP/mole of phospholipid.

DETD [0066] Two types of in vivo experiments were performed to determine the vasorelaxant and hypotensive effects of VIP in **liposomes** prepared by the two methods. In a first series of experiments, the bioactivity of VIP in the **liposome** preparations was examined as a function of vasodilation, while in the second series of experiments, the duration and efficacy of VIP in the two **liposome** preparations on mean arterial pressure was measured.

DETD [0067] In the first experiments, the bioactivity of VIP in the **liposome** preparations was measured as a function of change in arteriolar diameter in hamster cheek pouch. Adult male golden Syrian hamsters. . . pouch was first suffused with bicarbonate buffer during a 30 minutes equilibration period, and then with 1.4 ml of each **liposome** preparations described above for 7 minute.

DETD [0068] VIP in **liposomes** prepared by the first method, outside the scope of the invention, did not elicit an increase in arteriolar diameter significantly. . . [Suzuki, et al., Life Sci. 57(15):1451-1457 (1995)]. When this observation is compared to the previous observation that VIP in conventional **liposomes** prepared with the same method but without an extrusion step shown enhanced and prolonged effects in situ [Suzuki, et al.,. . . result is significant in demonstrating that SSL in general are not amenable to the present invention. VIP (0.1 nmol) in **liposomes** prepared by the second method

and within the scope of the invention, exhibited a significant increase in arteriolar diameter from. . . persisted for 9 to 16 minutes after suffusion was stopped. This result was more similar to previous observations using conventional **liposomes** [Suzuki, et al., Life Sci. 57(15):1451-1457 (1995)].

DETD [0069] In examining the duration and efficacy of VIP in the two **liposome** preparations on mean arterial pressure, the following procedure was carried out. Adult mate hamsters with spontaneous hypertension (n=12) were obtained from the Canadian Hybrid Farms (Hall Harbour, Nova Scotia, Canada). Approximately 500 μl each of three test preparations, **liposomes** prepared by the second method above, VIP in aqueous solution, and **liposomes** without VIP, were injected administered over the course of 1 minute in the femoral vein. Continuous anesthesia of the animals. . .

DETD [0070] After injection of 0.1 nmol **liposome**-associated VIP, a significant and gradual decrease in mean arterial pressure up to 50% was observed in the first 2.5 hours. . . period of the experiment as shown in FIG. 6. No significant effect on mean arterial pressure was observed using empty **liposomes** or VIP in aqueous solution. These data suggest that intravenously administered VIP in SSL successfully normalized the mean arterial pressure. . . to produce normal blood pressure was very low compared to previous observations wherein the same amount of VIP in conventional **liposomes** produced a 30% decrease in mean arterial pressure of normotensive hamsters [Gao, et al., Life Sci. 54:PL247-PL252 (1994)], but this. . .

DETD . . . method) retained the VIP activity, the results suggest that extrusion was responsible for the loss of bioactivity in the first **liposome** preparation. This possibility is consistent with a previous demonstration wherein interleukin-2 was shown to lose more than 25% activity after. . .

DETD [0072] For morphological evaluation of vesicle prepared by both methods described in Example 3, **liposomes** were prepared for freeze-fracture according to standard techniques as reported previously [Alkan-Onyuksel, et al., J. Pharm. Sci. In press (1996)]. Briefly, drops of each **liposome** suspension were frozen in liquid-nitrogen cooled Freon 22, fractured using a Balzers BAF 301 freeze-etch unit at -115° C., and. . .

DETD . . . Biol. 22:263-268 (1995)]. Possibly, the formation of larger vesicles may have promoted the entrapment of VIP molecules inside the final **liposomes**, while retaining a rather small mean size and distribution required for long circulation times.

DETD Peptide Activity in a Simplified **Liposome** Preparation

DETD . . . a simple method for producing SSL associated with a biologically active peptide is provided which acts to maintain the resulting **liposomes** at a size approximately less than 200 nm. In addition an alternative method of preparation was examined and the effects. . .

DETD [0075] Egg yolk **PC**, egg yolk PG, **cholesterol**, and PEG-DSPE were mixed in chloroform at a molar ratio of 5:1:3.5:0.5 and the solvent evaporated using a water bath. . . for 5 minutes and extruded through stacked polycarbonate filters using a LiposoFast apparatus. Human VIP was added to the resulting **liposomes** having an average diameter of less than 300 nm and the mixture incubated overnight at 4° C. Free VIP was separated from the VIP-associated **liposomes** using a Bio-gel A-5m column and collected **liposomes** stored under argon at 4° C. until use. Size of the **liposomes** determined by quasi electric light scattering indicated an average diameter of 162±59 nm. Phospholipid concentration and VIP recovery were determined. . .

DETD [0079] These results indicated that the dehydration /rehydration step described in Example 3 is not necessary to formation of active **liposome** preparations. More importantly, **liposomes** prepared by this method retained an average diameter of less than 200 nm and retained equal, if not higher, VIP activity than either **liposome** preparation described in Example 3. As an additional advantage, the VIP:phospholipid ratio which resulted from this preparative method was higher. . .

DETD [0081] **Liposomes** prepared as described in Example 3 were transferred

intravascular. . .

DETD [0082] Relative echogenicity (apparent brightness) of **liposome** formulations was objectively assessed by computer-assisted videodensitometry. The process involved acquisition, pre-processing, automated **liposome** identification, and gray scale quantification. Image processing and analysis were performed with Image Pro Plus Software (Ver. 1.0, Media Cybernetics,. . . running on a dedicated computer (486 CPU, 66 MHz). Randomly selected IVUS images were acquired from video tape for each **liposome** formulation. Images were digitized to 640×480 pixels spatial resolution (approximately 0.045 mm/pixel) and 8 bit (256 levels) amplitude resolution. all. . . scaled such that a reference feature, common to each image, retained a constant gray scale value over all images. An automated-**liposome** detection routine was then run to identify **liposomes** suspended in solution within an annular region of interest set at a constant radial distance from the imaging catheter. The automated **liposome** detection routine identified all "bright" objects within the analysis annulus having a gray scale level greater than 29, a roundness. . . 4 pixels. This procedure excluded virtually all imaging artifacts from the detection algorithm. Thus, object identified were considered to be "**liposomes**." Each **liposome** was outlined and numbered by the computer program. The average gray scale and size of each value of all pixels identified as "**liposomes**" with a given image was then computed and used to characterize the echogenicity of a given **liposome** formulation. The results of these experiments demonstrate that the acoustic reflectance of the VIP **liposome** preparation has a gray scale of 119 (on a gray scale of 0 to 255 with 255 as pure white and 0 as pure black). Larger **liposomes** produced using lyophilization methods described in PCT Publication WO 93/20802 are characterized by an acoustic reflectance of about 110-120 while **liposomes** comprising contrast media such as Albunex® have an acoustic reflectance of about 110-120. Accordingly, the invention provides small diameter **liposomes** while retaining their acoustic imaging properties.

DETD . . . this example, DSPE conjugated with 1, 2, 3 or 5 kDa PEG in solution, alone or mixed with egg yolk **phosphatidylcholine** (EYPC) were studied by static (SLS) and dynamic light scattering (DLS).

DETD [0100] According to this example the therapeutic uses of the invention are analyzed. Previously, sterically stabilized **liposomes** (SSL) were prepared with VIP non-covalently associated on their surface. In this example, the need to conjugate VIP covalently to. . .

DETD . . . 3400] and polyethylene glycol (M_w 2000) conjugated distearyl phosphatidylethanolamine (DSPE-PEG_{2000}) were obtained from Sheanvater Polymers, Inc. (Huntsville, Ala.). BODIPY-Chol (flourescent **cholesterol**) was obtained from Molecular Probes Inc. (Portland, Ore.). Fluo-VIP.TM. (Portland, Ore.). Fluo-VIP .TM. fluorescein labeled VIP) was purchased from Advanced. . . VIP (human/rat) was synthesized, using solid-phase synthesis by Protein Research Laboratory at Research Resources Center, University of Illinois at Chicago. Egg-**phosphatidylcholine** (**PC**) and **cholesterol** (CH) were obtained from Sygena (Switzerland). Virgin female Sprague-Dawley rats (.about.140 g body weight) were obtained from Harlan (Indianapolis, Ind.).

DETD [0105] For testing the in vitro binding, BODIPY-Chol (a non-exchangeable fluorescent probe) containing **liposomes**, were prepared with film rehydration-extrusion method, as described in S. Dagar et al., Pharm. Sci., 1:S-294 (1998) and M. Patel. . . Control. Rel. Bioact. Mat., 24:913-914 (1997) but incorporated the probe at 1:1500 molar ratio (lipid:probe) in the lipid mixture. Egg **phosphatidylcholine** (**PC**), **cholesterol** (CH), DSPE-PEG_{2000} and dipalmitoyl phosphatidylglycerol (DPPG) in the molar ratios of **PC**:DPPG:DSPE-PEG_{2000}:CH of 0.50:0.10:0.03:0.35 were used to form the sterically stabilized **liposomes** by film rehydration and reconstitution using isotonic, 0.01 M HEPES buffer (pH 6.6). This was followed by extrusion through polycarbonate filters (100 nm) using a Liposofast® (Avestin

as determined using quasi-elastic light scattering (NICOMP 370, Particle Sizing Systems, Santa Barbara, Calif.). DSPE-$PEG_{3400-VIP}$ was inserted into these fluorescent **liposomes** by overnight incubation at 4° C. to form fluorescent VIP conjugated sterically stabilized **liposomes** (VIP-SSL).

DETD . . . The VIP-SSL were compared to SSL without VIP or with non-covalently associated VIP and the difference in number of fluorescent **liposomes** present on the tissue indicated the difference in attachment of VIP-SSL to MNU-induced rat breast cancer tissues.

DETD [0108] In this experiment VIP was successfully conjugated to DSPE-PEG_{3400} and incorporated into preformed sterically stabilized **liposomes** to form a VIP-SSL construct. The results showed the feasibility of this novel construct to actively target to MNU-induced rat. . .

CLM What is claimed is:

. . . and AIDS-associated dementias, comprising the step of administering to an individual suffering from the disease state an amount of a **liposome** composition effective to alleviate conditions associated with the disease state, said **liposome** composition prepared by a method comprising the steps of: a) mixing a combination of lipids wherein said combination includes at least one lipid component covalently bonded to a water-soluble polymer; b) forming sterically stabilized **liposomes** from said combination of lipids; c) obtaining **liposomes** having an average diameter of less than about 300 nm; and d) incubating **liposomes** from step (c) with a biologically active amphipathic compound under conditions in which said compound becomes associated with said **liposomes** from step (c) in an active conformation, wherein at least one amphipathic compound is a member of the VIP/glucagon/secretin family. . .

2. The method according to claim 1 wherein said **liposome** composition comprises **unilamellar liposomes**.

3. The method according to claim 1 wherein said **liposome** composition comprise multivesicular **liposomes**.

4. The method of according to claim 3 wherein said multivesicular **liposomes** are produced by carrying out the steps of sequentially dehydrating and rehydrating **liposomes** obtained in step (c) with said biologically active peptide.

9. The method according to claim 1 wherein the **liposomes** obtained in step (c) have an average diameter or less than about 200 nm.

10. The method according to claim 9 wherein the **liposomes** obtained in step (c) have an average diameter or less than about 100 nm.

11. The method according to any one of claims 1, 8, or 9 wherein the **liposomes** are obtained in step (c) by extrusion to form **liposomes** having a selected average diameter.

12. The method according to any one of claims 1, 8, or 9 wherein the **liposomes** are obtained in step (c) by size selection.

. . . 13. The method according to claim 1 wherein the combination of lipids consists of distearoyl-phosphatidylethanolamine covalently bonded to PEG (PEG-DSPE), **phosphatidylcholine** (**PC**), and phosphatidylglycerol (PG) in further combination **cholesterol** (Chol).

14. The method according to claim 13 wherein the combination of lipids are combined with **cholesterol** in a PEG-DSPE:**PC**:PG:Chol molar ratio of 0.5:5:1:3.5.

L17 ANSWER 4 OF 15 USPATFULL on STN
2002:136589 Archaesomes, archaeosomes containing coenzyme Q10 and other types

of liposomes containing coenzyme Q10 adjuvants and as delivery vehicles.
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US 6403117 B1 20020611
APPLICATION: US 2000-612618 20000706 (9)
PRIORITY: US 1995-8724P 19951215 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Novel archaeosome compositions and their use in vaccine formulations as adjuvants and/or delivery systems, to enhance the immune response to immunogens in an animal such as a human, are described. Another aspect relates to the use of these archaeosomes to enhance the delivery of compounds such as pharmaceuticals to specific cell types and tissues in animals and other life forms, via various routes of administration such as subcutaneous, intramuscular, and oral. The efficacy of the archaeosomes and also of conventional **liposomes** can be further improved in these applications, by incorporation of coenzyme Q_{10} and/or polyethyleneglycol-lipid conjugate into **liposomes** made from these archaeosomes.

TI Archaesomes, archaeosomes containing coenzyme Q10 and other types of **liposomes** containing coenzyme Q10 adjuvants and as delivery vehicles

AB . . . via various routes of administration such as subcutaneous, intramuscular, and oral. The efficacy of the archaeosomes and also of conventional **liposomes** can be further improved in these applications, by incorporation of coenzyme Q_{10} and/or polyethyleneglycol-lipid conjugate into **liposomes** made from these archaeosomes.

SUMM This invention relates to **liposomes** (closed lipid vesicles) made from archaeobacterial lipids, from non-archaeobacterial lipids, and mixtures thereof, and to the use of such **liposomes** for the enhanced delivery of pharmaceutical and other compounds to specific cell types such as macrophages/phagotocytes/antigen processing cells and to. . .

SUMM **Liposomes** are closed lipid vesicles containing an entrapped aqueous volume. The hydrophilic head groups of the lipids forming **liposomes** are oriented towards the aqueous environments present inside and outside the **liposomes**, whereas the hydrophobic regions of the lipids are sandwiched between the polar head groups and away from the aqueous environments. **Liposomes** may be **unilamellar** containing a single lipid bilayer, or multilamellar containing multiple bilayers (onion-like in structure) with an aqueous space separating each bilayer from the other. Various techniques for forming **liposomes** have been described in the literature, including but not limited to, pressure extrusion, detergent dialysis, dehydration-rehydration, reverse-phase evaporation, remote loading, sonication and other methods (13). **Liposomes** made from conventional ester phospholipids such as **phosphatidylcholine** are referred to herein as conventional **liposomes**, even if they contain sterols or other compounds in their bilayer.

SUMM **Liposomes** consisting of a lipid bilayer, a monolayer or a combination thereof, made from any lipid(s) which include in their composition. . .

SUMM . . . vary and may include phospho groups, glyco groups, phosphoglyco groups, polyol groups, c hydroxyl groups (18). In contrast to the **phosphatidylcholine** conventional lipid commonly used in **liposome** formulations, the phosphocholine head group is very rarely found in archaeobacterial polar lipids. Archaea provide a large selection of lipids. . .

SUMM There is much interest in the use of **liposomes** for medical, pharmaceutical, and other commercial applications. Most of the research reported on **liposomes** to-date, has been conducted using conventional phospholipids sometimes mixed with sterols (e.g., **cholesterol**) or other compounds to improve stability, rather than using either archaeobacterial or non-archaeobacterial ether lipids.

SUMM In a comparative study on the uptake of **liposomes** made with 1,2-diacyl-sn-glycero-3-phosphocholine and its ether analog, by cultured rat liver hepatocytes, the cellular uptake of both **liposome** types was

found to be similar (21). In another study, **liposomes** made with ether dipalmitoyl **phosphatidylcholine** or its ether analogue 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine, were phagocytosed at about the same rate by J774.E1 macrophage cells (6). Therefore, from these disclosures it would be expected that **liposomes** made with ether lipids, including by extension those from ether lipids either extracted from Arcaea or from ether lipids chemically. . . . lipid structures of Archaea, would be taken up by certain cells such as macrophages, to a similar extent as conventional **liposomes**. However, the current invention proves to the contrary, showing enhanced phagocytosis of vesicles (archaeosomes) made with archaeobacterial ether lipids.

SUMM . . . types such as macrophages is a current problem, e.g., the bacterium Mycobacterium tuberculosis which causes tuberculosis, viruses such as the **human immunodeficiency virus** (**HIV**) which causes acquired immune deficiency syndrome (AIDS), and parasites which cause malaria. A superior uptake of archaeosomes made with ether. . . .

SUMM There is considerable interest in the potential use of **liposomes** in the field of vaccine applications. **Liposomes** prepared from conventional phospholipids, sometimes mixed with **cholesterol** or other compounds (conventional **liposomes**) have been tested as potential antigen carriers/vehicles. Allison and Gregoriadis (1) reported that **liposomes** prepared from egg **phosphatidylcholine** had some adjuvant activity, provided a negatively charged lipid was included in the **liposome** composition. Since conventional **liposomes** often demonstrate only small adjuvant effects as compared with administration of the free antigen, various immunostimulatory substances such as lipid A have been co-incorporated into the **liposomes**, together with the antigen (4). However, as is the case with lipid A and Freund's adjuvant, immunostimulatory substances may have. . .

SUMM The humoral immune response, in mice, to bovine serum albumin encapsulated in **liposomes** made with dialkyl-ether sn-3-**phosphatidylcholine** was lower than that obtained with similar **liposomes** made with diacyl-ester sn-3 **phosphatidylcholine** (17). There is no teaching in the prior art to suggest true compared with **liposomes** made using conventional phospholipids, those made using archaeobacterial ether lipids would have a superior adjuvant effect in stimulating the immune. . .

SUMM . . . been shown to enhance the in vivo phagocytic activity in animal models (3). Labelled CoQ_{10} has been used in conventional **liposomes** as a marker for myocardial imaging and for studying tissue distribution of conventional **liposomes** coated with polysaccharides (7,22). In **liposomes**, CoQ_{10} is associated with the lipid layer of the vesicles. However, none of these or other prior art publications teach. . . to co-administered immunogen(s) (current claimed invention). Similarly, the prior art does not teach that the combination of CoQ_{10} in conventional **liposomes** would increase the phagocytosis of the resultant **liposomes** by macrophages, or allow for the alteration of tissue targeting profiles when the **liposomes** are administered to an animal by different routes, or that liposomal CoQ_{10} combination would enhance the immune response to co-administered. . .

SUMM It is another object of the invention to incorporate coenzyme Q_{10} into archaeosomes, or into **liposomes** prepared exclusively from lipids other than archaeobacterial-like ether lipids, to enhance the phagocytosis of the respective archaeosomes/**liposomes**, and/or to enhance the delivery of CoQ_{10} as well as other associated drug(s), and to enhance the immune response to an antigen associated with the respective archaeosomes/**liposomes**.

SUMM It is another object of this invention to incorporate CoQ_{10} into archaeosomes and conventional **liposomes**, sometimes in combination with polyethylene glycol lipid conjugates, to increase the delivery of various associated compounds to specific organ tissues. . . have been sterically stabilized by association with polyethyleneglycol conjugates, would therefore further increase the utility of archaeosomes and of

conventional **liposomes**, for delivery of compounds, including immunogens and CoQ_{10} itself, to phagocytic cells and to specific tissues.

DRWD FIG. 1. Fluorescence micrographs of cells incubated with Methanosarcina mazei archaeosomes, or with conventional **liposomes** prepared from DMPC:DMPG:CHOL, each vesicle type containing CF. Panels A_1, B_1, C_1, murine peritoneal macrophages. Panels A_2, B_2, C_2, J774A.1 macrophages. Panels A3, B_3, C_3, HEp-2 cells. Panels A_{1-3}, archaeosomes; Panels B_{1-3}, conventional **liposomes**; and panels C_{1-3}, cells without added vesicles. The presence of yellow-fluorescent **liposomes** are indicated in the black and white photos by light areas. Magnification bar=20 μm.

DRWD . . . of murine peritoneal macrophages, J774A.1, and HEp-2 cells incubated with multilamellar M. mazei archaeosomes (A_1, B_1, C_1), or multilamellar conventional **liposomes** prepared from DMPC:DMPG:CHOL (A_2, B_2, C_2), each vesicle type containing CF. Panels A_{1-2}, J774A.1 cells. Panels B_{1-2}, murine peritoneal macrophages.. . .

DRWD FIG. 3. Tissue distribution of (A) conventional **liposomes** (DSPC:DCP:CHOL) and, (B) $CoQ_{10\text{-conventional}}$ **liposomes** (DSPC:DCP:CHOL:Q_{10}) 24 hours after oral and parenteral administration to mice. The data shown are ± sample standard error from the. . .

DRWD FIG. 4. Tissue distribution of (A) PEG-conventional **liposomes** (DSPC:DCP:CHOL:DSPE-PEG) and, (B) PEG-$CoQ_{10\text{-conventional}}$ **liposomes** (DSPC:DCP:CHOL:DSPE-PEG:Q_{10}) 24 hours after oral and parenteral administration to mice. The data shown are ± sample standard error from the. . .

DRWD . . . M. smithii (0.57 mg); M. voltae (1.83 mg); M. hungatei (1.09 mg); M. concilii (0.68 mg); M. stadtmanae (0.51 mg); **PC**:PG (DMPC:DMPG, 2.11 mg); **PC**:PG:CHOL (DMPC:DMPG:CHOL, 0.12 mg); and **PC**:DCP:CHOL (DMPC:DCP:CHOL, 0.87 mg). Data are the means from mice in duplicates.

DETD The inventors have discovered that archaeosomes are taken up by phagocytic cells to a greater extent than are conventional **liposomes**. Another aspect of the invention shows the improved uptake by phagocytic cells of both conventional **liposomes** and of archaeosomes, through the incorporation of coenzyme Q_{10} in the respective vesicles. Incorporation of CoQ_{10} into conventional **liposomes**, and archaeosomes, also allows for the improved targeting of vesicles to specific tissues in the animal, for vesicles administered via. . . be especially applicable for oral delivery of vaccines. Further, archaeosomes in general, are shown to be superior, compared to conventional **liposomes**, as carriers of antigens, resulting in improved immune response to the antigen administered to an animal such as a human. Further, it is shown that archaeosomes act as superior adjuvants, compared to conventional **liposomes**, resulting in an increased immune response to an antigen administered to an animal such as a human. Also, the duration. . . used in the preparation of vesicles, is shown to improve the immune response to an antigen co-entrapped either in conventional **liposomes** and/or in archaeosomes.

DETD . . . backbone via ether bonds; archaeal or archaeobacterial lipid(s), lipid(s) derived from a member(s) of the class Archaea (synonymous to Archaeobacteria); **liposome**, closed vesicle made of lipid bilayer membranes which entrap an aqueous volume, the **liposome** may be **unilamellar** (one bilayer) or multilamellar (multiple bilayers, each separated from the adjoining one by aqueous spaces); conventional **liposome**, a **liposome** made with conventional phospholipids and in some cases including a sterol and may include other compounds that are entrapped within. . . lipids), or a combination of mono and bilayers (if made with diether or other monpolar lipids and tetraether lipids); vesicle, **liposome** or archaeosome; bare antigen, antigen without adjuvant or vesicle; bare **liposome**/archaeosome, **liposome** or

archaeosome matrix an associated antigen, adjuvant, a substance of material which when administered with an immunogen increases the immune. . .

DETD L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dimyristoylphosphatidylglycerol (DMPG), distearoylphosphatidylcholine (DSPC), dicetylphosphate (DCP, i.e., dihexadecyl phosphate), **cholesterol** (CHOL) (all these were at least 99% pure), 5(6)-carboxyfluorescein (CF), Triton X-100, n-propyl gallate, peroxidase substrate 2,2'-azino-bis (3-ethylbenzthiazoline sulphonic acid),. . .

DETD **Liposome** and Archaeosome Preparation

DETD For **liposome** and archaeosome preparation, the lipids dissolved in chloroform were dried under a stream of N_2, and placed in a lyophilizer. . . consisted of 0.5 ml of 10 mM potassium phosphate buffer, pH 7.14, containing 160 mM NaCl (PBS). Unless indicated otherwise, **liposomes** and archaeosomes were made by pressure extrusion of the hydrated lipids through two stacked 400 nm filters in a LiposoFast apparatus similar to that described by MacDonald et al. (10) to obtain predominantly **unilamellar** vesicles. Archaeobacterial lipids were hydrated for 1-2 hours, or sometimes overnight, at 35° C. and the resulting multilamellar vesicles were. . . ambient temperature. Conventional phospholipids were hydrated (1-2 h) and extruded at 50° C. for DPPC, and at 35° C. for DMPC:DMPG:**cholesterol** (1.8:0.2:1.5, molar ratio) or DMPC:DMPG (1.8:0.2, molar ratio) to obtain **unilamellar** vesicles.

DETD The in vitro uptake of archaeosomes prepared from the TPL extracted from M. mazei and of conventional **liposomes** prepared from DPPC:CHOL (5:5 molar ratio), DSPC:CHOL (5:5 molar ratio) or DSPC:CHOL:DCP (4:5:1 molar ratio) was also studied using cholesteryl. . . required, coenzyme Q_{10} dissolved in chloroform was added (at 5:20 weight/weight ratio of total lipids) before drying the lipids. The **liposomes** and archaeosomes were prepared by the reverse-phase evaporation (REV) method (at 55° C.) combined with bath sonication (at room. . .

DETD Conventional **liposomes** (DSPC:DCP:CHOL at a molar ratio of 4:1:5), $CoQ_{10\text{-conventional}}$ **liposomes** (DSPC:DCP:CHOL:CoQ_{10} at a molar ratio of 3:1:4:2), PEG-conventional **liposomes** (DSPC:DCP:CHOL at a molar ratio of 4:1:5 plus DSPE-PEG at 7% molar ratio of the total lipids), and PEG-$CoQ_{10\text{-conventional}}$ **liposomes** (DSPC:DCP:CHOL:CoQ_{10} at a molar ratio of 3:1:4:2 plus DSPE-PEG at 7% molar ratio of the total lipids) were prepared with. . .

DETD . . . archaeosomes (prepared from the TPL, or a lipid obtained in a biologically pure form from the indicated archaeobacterium) and conventional **liposomes** (prepared from DMPC:DMPG at 1.8:0.2 molar ratio, or DMPC:DMPG:CHOL at 1.8:0.2:1.5 molar ratio, or DMPC:DCP:CHOL at 7:1:2 molar ratio) containing. . .

DETD It will be understood by one skilled in the art that the preparation of **liposomes** and archaeosomes of this invention and the association/incorporation of other compounds in the respective vesicles is not limited to the. . .

DETD Peroxidase activity in 5 to 35 μg dry weight of **liposomes** or archaeosomes was assayed in 1 ml reaction mixtures containing 45 mM citric acid, pH 4.0, 2.2 mM H_{2O2}, 0.2. . .

DETD The amount of protein incorporated into or associated with archaeosomes and **liposomes** to be used for immunizations was quantitated by SDS polyacrylamide gel electrophoresis (PAGE), wherein the lipids separate from the protein. . . The amount of protein incorporated/associated with the vesicles was compared on the basis of salt free dry weights of the **liposomes** or archaeosomes, respectively.

DETD Archaeosomes or **liposomes** containing encapsulated peroxidase were incubated with different cell types to quantitate the respective vesicle binding to mammalian cells. Peritoneal macrophages. . .

DETD Fluorescence microscopy was performed to visualize archaeosome or **liposome** binding to several cell types. For these studies, CF was entrapped within the vesicles at a concentration of only 1.5. . .

DETD These uptake studies were performed essentially as described above, but

used liposomes and archaeosomes prepared to contain entrapped cholesteryl [1-^{3H}] ether ($^{3H\text{-chol}}$) as a tracer. One micro curie (1 μCi=37 kBq) of. . .

DETD . . . each antigen/adjuvant preparation three mice were injected (unless indicated otherwise) i.p., i.m., or s.c. with antigens encapsulated in archaeosomes or **liposomes** (diluted in sterile PBS, pH 7.1), antigens emulsified in CFA, or with antigens diluted in PBS alone (final volume, 0.2 ml/mouse). For the second immunization, mice were injected with antigens encapsulated in archaeosomes or **liposomes**, or emulsified in IFA, or diluted in PBS. For experiments where a third or fourth injection was required, antigens were. . .

DETD Construction and Characterization of Archaeosomes and **Liposomes**

DETD Peroxidase encapsulated within vesicles was used to quantitate the relative uptake of the various types of archaeosomes and of conventional **liposomes**, by eukaryotic cells. The amounts of each vesicle type taken up by adherent cells seeded onto culture wells could be. . .

DETD Archaeosomes were prepared from the TPL extracted from several archaeobacteria, and conventional **liposomes** prepared from two conventional phospholipid formulations that have been frequently used in the prior art (2, 17). The percentage of. . .

DETD . . . the data in FIG. 2, we prepared intermediate-sized vesicles of approximately 200 nm diameter by pressure extrusion of hydrated multilamellar **liposomes** through filters of 400 nm pore size. This resulted in populations of vesicles with the size range distributions defined in Table 1. The distributions were quite narrow except for DPPC **liposomes**, which consisted of two differently sized populations.

DETD Binding of Archaeosomes and Conventional **Liposomes** to Cells

DETD . . . Peroxidase and fluorescence assays were used to quantitate and compare the binding of several types of archaeosomes and of conventional **liposomes** to two phagocytic and three non phagocytic cell lines. The results are illustrated in FIG. 1, and in Tables 2. . . uptake of archaeosomes by the two macrophage lines (murine peritoneal and J774A.1 macrophages) was several times greater than the conventional **liposomes**. Compared with macrophage cell lines, the uptake of archaeosomes and of conventional **liposomes** by non-phagocytic cell lines (Hep-2, HeLa, EJ/28) was substantially lower.

DETD Large, multilamellar vesicles (0.5 to 3 μm) prepared from the polar lipids of M. mazei or from DMPC:DMPG:**cholesterol** gave trends similar to those obtained with the smaller vesicles, i.e. greater uptake of archaeosomes than conventional **liposomes** by murine peritoneal macrophages, and by J774A.1 cells; lesser uptake of both vesicle types by the non- phagocytic HEp-2 cell. . .

DETD These data, using encapsulated peroxidase or fluorescent dye as markers, illustrate the enhanced phagocytosis of archaeosomes compared with conventional **liposomes**. The potential advantages for using archaeosomes for delivery of compounds to macrophages is discussed elsewhere in this submission.

DETD . . . inhibition of phagocytosis) of the macrophages over the time periods studied. This indicates that the inhibitors prevented internalization of the **liposomes** into the macrophages. In addition, the time-dependent decline in fluorescence upon readjustment of the temperature of the culture medium from. . .

DETD . . . into archaeosomes prepared from the TPL of M. mazei, which are known to be anionic (19), and into anionic conventional **liposomes** (DSPC:CHOL:DCP), with relatively high entrapment efficiencies (Table 5). However, compared with anionic lipid mixtures, even higher entrapment efficiencies were obtained. . .

DETD The uptake by macrophages, of archaeosomes and of conventional **liposomes** lacking coenzyme Q_{10} is shown as a function of time using $^{3H\text{-chol}}$ as the tracer marker (Table 6A). At 37°. . . indicated times, it can be seen that M. mazei archaeosomes are taken up substantially better than all formulations of conventional **liposomes**, as was also shown in Table 2. The data in Table 6A serve as control values to assess the effect. . . contained coenzyme Q_{10}. This enhancing effect of coenzyme Q_{10} was several times higher with the

archaeosomes with the conventional **liposomes**. Comparable profiles of accumulation and the enhancing effects of coenzyme Q_{10} entrapment on the uptake of archaeosomes and of conventional **liposomes**, was observed at all lipid concentrations tested (compare Tables 7A and 7B).

DETD . . . clearly indicate the potential for inclusion of coenzyme Q_{10} into the vesicles for increased targeting of archaeosomes and of conventional **liposomes** to various cell types. An increased uptake of vesicles containing coenzyme Q_{10} by macrophages (antigen processing cells, and the sites. . . drugs (including antiviral and antimicrobial agents). Another application is to deliver the water-insoluble drug (coenzyme Q_{10}) to mammalian cells, via **liposomes** and archaeosomes.

DETD The tissue distribution profiles of label from conventional **liposomes** and $CoQ_{10\text{-conventional}}$ **liposomes** administered by various routes showed that these were generally similar (FIGS. 3A and 3B). However, the major difference was that in orally administered $CoQ_{10\text{-conventional}}$ **liposomes**, no accumulation of the label (at 24 h) was seen in any of the tissues examined, except for in the. . .

DETD . . . reticuloendothelial system (predominantly present in the spleen and liver), and hence prolong the circulation half life in the blood. Such **liposomes** have been called sterically stabilized **liposomes** (24). The tissue distribution profile of PEG-conventional **liposomes** (FIG. 4A) was somewhat similar to that of conventional **liposomes** (FIG. 3A). However, A was surprising to see that when CoQ_{10} was incorporated into the PEG-conventional **liposomes** which were administered orally, there was an increased accumulation of the marker (24 hours post administration) into spleen, liver, intestine,. . . The combined accumulation in the spleen and liver was about 8-fold greater. With the i.v. route of administration, the PEG-CoQ_{10}-conventional **liposomes** showed no presence of the marker in the spleen (FIG. 4B).

DETD . . . the intestine and kidneys, the tissue distribution profiles of M. mazei archaeosomes (FIG. 5A) showed differences from those with conventional **liposomes** (FIG. 3A). When M. mazei archaeosomes were administered via i.m., s.c., or i.v. routes, the tissue distribution profile (at 24. . .

DETD . . . the liver and spleen, was about 5-fold better than the highest level obtained (FIG. 4B) with any combination using conventional **liposomes**. For i.m. and i.v. administered PEG-$CoQ_{10\text{-archaeosomes}}$, the accumulation of label in the spleen was significantly lower than that obtained in. . .

DETD The 48 hour tissue distribution profile of label from orally administered PEG-CoQ_{10} conventional **liposomes** was similar to that observed at 24 hours (FIG. 4B). However, the 48 hour tissue distribution profile of label from. . .

DETD The data in these figures show that a significant enhancement in the efficacy of the delivery/accumulation of orally administered conventional **liposomes**, to the spleen and liver (the major sites for the antigen processing cells of the immune system), can be achieved. . .

DETD The enhanced uptake of archaeosomes by phagocytic cells, compared to that of conventional **liposomes**, suggested that archaeosomes may be superior as adjuvants and/or carriers of antigens for raising an immune response to an immunogen.. . .

DETD A comparison of the adjuvant/antigen carrier properties of archaeosomes and of conventional **liposomes** was made using BSA as the antigen (FIG. 8). The immune response to BSA was markedly enhanced when it was. . . and the results were again comparable, in some cases, to that achieved with Freund's adjuvant. In contrast, all three conventional **liposome** types yielded substantially lower immunostimulatory effects.

DETD The small adjuvant effect observed with DMPC:DMPG conventional **liposomes** can be improved by the inclusion of increasing amounts of archaeobacterial lipids in the lipids used for vesicle formation. This.

. .

DETD Mice were immunized (days 0 and 14), i.p., with conventional **liposomes** (DSPC:DCP:CHOL) containing 15 μg BSA per dose, with and without Q_{10} incorporated (see Table 5 and 6B for compositions). Sera. . . 18 when titred for antibodies (IgG+IgM) had a significantly higher anti-BSA antibody titre (at the 95% confidence limit) when the **liposomes** contained CoQ_{10}.

DETD . . . dialkyl-ether phosphatidylcholines, with BSA as the entrapped antigen, were less efficient in eliciting an immune response in mice compared to **liposomes** made with the diacyl-ester **phosphatidylcholine**. In addition to enhanced adjuvant effects, few boosts are required with archaeosomes. Moreover, the storage stability (shelf life) of archaeosomes. . .

DETD In vitro cytotoxicity studies with several phagocytic and non phagocytic cell lines described earlier indicated that neither archaeosomes nor conventional **liposomes** had any significant adverse effects, as assessed from cell viability assays, even when the vesicles were tested at lipid concentrations. . .

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DETD 20. Sprott, G. D., Patel, G. B., Choquet, C. G., and Ekiel, I. (1993) Formation of stable **liposomes** from lipid extracts of Archaeobacteria (Archaea). International Application No. PCT/CA92/00464, International Publication Number WO 93/08202.

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DETD 22. Takada, M., Yuzuriha, T., Katayama, K., Iwamoto, K., and Sunamoto, J. (1984) Increased lung uptake of **liposomes** coated with polysaccharides. Biochim. Biophys. Acta, 802: 237-244.

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DETD

Characteristics of peroxidase-archaeosomes and peroxidase-**liposomes** used in binding assays.[1]

Mean Diameter
Lipid source for (nm ± standard Peroxidase Activity[2]
vesicles deviation) No Detergent OGP

M. mazei 189 ±. . . Methanococcus voltae, and Thermoplasma acidophilum.
[2]Activity is expressed as change in absorbency/min/mg dry wt vesicles.
[3]Consisting of two populations of **liposomes** of 324 nm (84%, number weighted) and 2833 nm (16%, number weighted).
DETD
TABLE 2

Comparative in vitro uptake (μg/mg cell protein) of archaeosomes and conventional
liposomes to murine peritoneal macrophages and a variety of cell lines.[1]

Murine
Lipid source for Peritoneal J774A.1 EJ/28
vesicles Macrophages Macrophages HEp-2. . .
DETD
TABLE 3

Relative uptake of archaeosomes and conventional **liposomes** by various cell lines.[1]

Vesicles
Mean Diameter added per Relative Binding[3]
Lipid source for (nm ± standard assay well J774A.1. . .
DETD
TABLE 4

The effect of inhibitors on the phagocytosis of M. hungatei archaeosomes by J774A.1 macrophages.[1]

Relative binding/degradation of **liposomes** by
macrophages at different intervals (min)[2]
Treatment 0 30 60 120 180 240 300 360

None 10 8 5 3 2 1. . .
DETD
TABLE 5

Incorporation of coenzyme Q_{10} into M. mazei archaeosomes and conventional **liposomes**.[1]

Starting ratio Q_{10} Loading
Q_{10}:lipid Entrapment Ratio
Lipid source for vesicles (mg:mg) (%) (mg $Q_{10/mg}$ lipid)

M. mazei 5:20 62.1 ± 1.1. . .
DETD
TABLE 6A

Uptake of M. mazei archaeosomes and conventional **liposomes** by J774A.1 macrophages
as a function of time, using $^{3H\text{-}chol}$ as the marker.[1]

Lipid source for Uptake (cpm)[2]
vesicles 15 min 30 min. . .
DETD
TABLE 6B

Influence of coenzyme Q_{10} on the uptake of M. mazei archaeosomes and conventional
liposomes by J774A.1 macrophages as a function of time, using $^{3H\text{-}chol}$ as a marker.[1]

Lipid source for uptake (cpm)
vesicles 15 min 30. . .
DETD
TABLE 7A

Uptake of M. mazei archaeosomes and conventional **liposomes** by J774A.1 macrophages
as a function of lipid concentration, using $^{3H\text{-}chol}$ as a marker.[1]
Lipid source for Lipid concentration
vesicles 25 μM 50. . .
DETD
TABLE 7B

Influence of coenzyme Q_{10} on the uptake of M. mazei archaeosomes and conventional
liposomes by J774A.1 macrophages as a function of lipid concentration,
using $^{3H\text{-}chol}$ as a marker.[1]
Lipid source for Lipid concentration
vesicles 25 μM 50. . .
DETD . . . per injection i.p. to each mouse. Immunizations where antigen was not encapsulated consisted of BSA in PBS followed by bare **liposomes** 4 hours later. Bare BSA is a control for antigen alone in PBS. Immunizations were given at 0 and 14. . .
DETD . . . 91.4 ± 5.9
Archaeosomes 50 186 ± 57 0.86 44.0 ± 11.5
Archaeosomes 10 152 ± 54 1.14 22.4 ± 3.9
Liposomes 0 126 ± 56 1.01 9.8 ± 0.9

[1]Mice (3 per group) were immunized by i.p. injections of various adjuvants containing. . .
CLM What is claimed is:

1. A **liposome** composition comprising (1) the total polar lipids extract of an archaeobacterium, (2) a pharmaceutical agent and (3) coenzyme Q_{10}.

2. A **liposome** composition according to claim 1, further comprising (4) a polyethyleneglycol lipid conjugate.

3. A **liposome** composition according to claim 1, wherein the archaeobacterium is selected from the group consisting of Methanosarcina mazei, Methanospirillum hungatei, Methanosphaera. . .

4. A **liposome** composition according to claim 1, wherein the **liposome** is **unilamellar**.

5. A **liposome** composition according to claim 1, wherein the **liposome** size is in the range of not less than 50 nm, but less than 500 nm, in diameter.

6. A **liposome** composition according to claim 1 wherein the archaeobacterium is Methanosarcina mazei.

7. A **liposome** composition according to claim 1, wherein the archaeobacterium is Thermoplasma acidophilum.

8. A method for the delivery of a pharmaceutical agent to an animal, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archaeobacterium and coenzyme Q_{10}, as a. . .

9. A method for the delivery of a pharmaceutical agent to an animal, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archaeobacterium, coenzyme Q_{10}, and a polyethyleneglycol. . .

10. A method according to claim 9, wherein the **liposome** is administered to an animal orally, intraperitoneally, intramuscularly, subcutaneously, or intravenously.

. . . method for enhancing the targeted delivery of a pharmaceutical agent to specific animal organs, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archaeobacterium, coenzyme Q_{10}, and a polyethyleneglycol. . .
13. A method according to claim 12,wherein the **liposome** is delivered to an animal via the oral route.

. . . the delivery of a pharmaceutical or biological agent to phagocytic cells of an animal, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archacobacterium as a carrier for said. . .
. . . for a disease caused by a pathogen residing inside the cells of an animal, comprising administering to the animal a **liposome** prepared from the total polar lipids extract of an archaeobacterium and an antimicrobial or an antiviral agent.

17. A method of imaging of tissues and organs in an animal, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archaeobacterium as a carrier for an. . .
. . . selective delivery of a pharmaceutical or biological agent to specific tissues of an animal, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archaeobacterium and as a carrier for. . .
20. A method according to claim 18, wherein the **liposome** composition additionally comprises a polyethyleneglycol lipid conjugate.

. . . A method according to any one of claims 14, 15, 16, 17, 18, 19 or 20, wherein the dosage of **liposomes** to be delivered is 4 to 73 mg/kg of animal body weight.

24. A method according to any one of claim 14, 15, 16, 17, 18, 19 or 20, wherein the **liposome** is administered to an animal orally, intraperitoneally, intramuscularly, subcutaneously, or intravenously.

L17 ANSWER 5 OF 15 USPATFULL on STN
2000:137879 Archaeosomes, archaeosomes containing coenzyme Q_{10}, and other types of **liposomes** containing coenzyme Q_{10} as adjuvants and as delivery vehicles.
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Patel, Girishchandra B., Nepean, Canada
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National Research Council of Canada, Ottawa, Canada (non-U.S. corporation)
US 6132789 20001017
WO 9722333 19970626
APPLICATION: US 1998-77956 19980612 (9)
WO 1996-CA835 19961213 19980612 PCT 371 date 19980612 PCT 102(e) date
PRIORITY: US 1995-8724P 19951215 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB Novel archaeosome compositions and their use in vaccine formulations as adjuvants and/or delivery systems, to enhance the immune response to immunogens in an animal such as a human, are described. Another aspect relates to the use of these archaeosomes to enhance the delivery of compounds such as pharmaceuticals to specific cell types and tissues in animals and other life forms, via various routes of administration such as subcutaneous, intramuscular, and oral. The efficacy of the archaeosomes and also of conventional **liposomes** can be further improved in these applications by incorporation of coenzyme Q_{10} and/or polyethyleneglycol-lipid conjugate into **liposomes** made from

these archaeosomes.

TI Archaeosomes, archaeosomes containing coenzyme Q_{10}, and other types of **liposomes** containing coenzyme Q_{10} as adjuvants and as delivery vehicles

AB . . . via various routes of administration such as subcutaneous, intramuscular, and oral. The efficacy of the archaeosomes and also of conventional **liposomes** can be further improved in these applications by incorporation of coenzyme Q_{10} and/or polyethyleneglycol-lipid conjugate into **liposomes** made from these archaeosomes.

SUMM This invention relates to **liposomes** (closed lipid vesicles) made from archaeobacterial lipids, from non-archaeobacterial lipids, and mixtures thereof, and to the use of such **liposomes** for the enhanced delivery of pharmaceutical and other compounds to specific cell types such as macrophages/phagocytes/antigen processing cells and to. . .

SUMM **Liposomes** are closed lipid vesicles containing an entrapped aqueous volume. The hydrophilic head groups of the lipids forming **liposomes** are oriented towards the aqueous environments present inside and outside the **liposomes**, whereas the hydrophobic regions of the lipids are sandwiched between the polar head groups and away from the aqueous environments. **Liposomes** may be **unilamellar** containing a single lipid bilayer, or multilamellar containing multiple bilayers (onion-like in structure) with an aqueous space separating each bilayer from the other. Various techniques for forming **liposomes** have been described in the literature, including but not limited to, pressure extrusion, detergent dialysis, dehydration-rehydration, reverse-phase evaporation, remote loading, sonication and other methods (13). **Liposomes** made from conventional ester phospholipids such as phosphatidyleholine are referred to herein as conventional **liposomes**, even if they contain sterols or other compounds in their bilayer.

SUMM **Liposomes** consisting of a lipid bilayer, a monolayer or a combination thereof, made from any lipid(s) which include in their composition. . .

SUMM . . . vary and may include phospho groups, glyco groups, phosphoglyco groups, polyol groups, or hydroxyl groups (18). In contrast to the **phosphatidylcholine** conventional lipid commonly used in **liposome** formulations, the phosphocholine head group is very rarely found in archaeobacterial polar lipids. Archaea provide a large selection of lipids. . .

SUMM There is much interest in the use of **liposomes** for medical, pharmaceutical, and other commercial applications. Most of the research reported on **liposomes** to-date, has been conducted using conventional phospholipids sometimes mixed with sterols (e.g., **cholesterol**) or other compounds to improve stability, rather than using either archaeobacterial or non-archaeobacterial ether lipids.

SUMM In a comparative study on the uptake of **liposomes** made with 1,2-diacyl-sn-glycero-3-phosphocholine and its ether analog, by cultured rat liver hepatocytes, the cellular uptake of both **liposome** types was found to be similar (21). In another study, **liposomes** made with either dipalmitoyl **phosphatidylcholine** or its ether analogue 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine, were phagocytosed at about the same rate by J774.E1 macrophage cells (6). Therefore, from these disclosures it would be expected that **liposomes** made with ether lipids, including by extension those from ether lipids either extracted from Archaea or from ether lipids chemically. . . lipid structures of Archaea, would be taken up by certain cells such as macrophages, to a similar extent as conventional **liposomes**. However, the current invention proves to the contrary, showing enhanced phagocytosis of vesicles (archaeosomes) made with archaeobacterial ether lipids.

SUMM Although the prior art e.g. in reference (20) and (25 to 29) does disclose **liposome** formation from certain archaeobacterial lipids and lipid fractions, there is no disclosure of the formation of **liposomes** from the **liposome** compositions claimed in this application.

SUMM . . . types such as macrophages is a current problem, e.g., the bacterium Mycobacterium tuberculosis which causes tuberculosis, viruses such as the **human immunodeficiency virus** (**HIV**) which causes

acquired immune deficiency syndrome (AIDS), and parasites which cause malaria. A superior uptake of archaeosomes made with ether. . .

SUMM There is considerable interest in the potential use of **liposomes** in the field of vaccine applications. **Liposomes** prepared from conventional phospholipids, sometimes mixed with **cholesterol** or other compounds (conventional **liposomes**) have been tested as potential antigen carriers/vehicles. Allison and Gregoriadis (1) reported that **liposomes** prepared from egg **phosphatidylcholine** had some adjuvant activity, provided a negatively charged lipid was included in the **liposome** composition. Since conventional **liposomes** often demonstrate only small adjuvant effects as compared with administration of the free antigen, various immunostimulatory substances such as lipid A have been co-incorporated into the **liposomes**, together with the antigen (4). However, as is the case with lipid A and Freund's adjuvant, immunostimulatory substances may have. . .

SUMM The humoral immune response, in mice, to bovine serum albumin encapsulated in **liposomes** made with dialkyl-ether sn-3-**phosphatidylcholine** was lower than that obtained with similar **liposomes** made with diacyl-ester sn-3 **phosphatidylcholine** (17). There is no teaching in the prior art to suggest that compared with **liposomes** made using conventional phospholipids, those made using archaeobacterial ether lipids would have a superior adjuvant effect in stimulating the immune. . .

SUMM . . . been shown to enhance the in vivo phagocytic activity in animal models (3). Labelled CoQ_{10} has been used in conventional **liposomes** as a marker for myocardial imaging and for studying tissue distribution of conventional **liposomes** coated with polysaccharides (7,22). In **liposomes**, CoQ_{10} is associated with the lipid layer of the vesicles. However, none of these or other prior art publications teach. . . to co-administered immunogen(s) (current claimed invention). Similarly, the prior art does not teach that the combination of CoQ_{10} in conventional **liposomes** would increase the phagocytosis of the resultant **liposomes** by macrophages, or allow for the alteration of tissue targeting profiles when the **liposomes** are administered to an animal by different routes, or that liposomal CoQ_{10} combination would enhance the immune response to co-administered. . .

SUMM It is another object of the invention to incorporate coenzyme Q_{10} into archaeosomes, or into **liposomes** prepared exclusively from lipids other than archaeobacterial-like ether lipids, to enhance the phagocytosis of the respective archaeosomes/**liposomes**, and/or to enhance the delivery of CoQ_{10} as well as other associated drug(s), and to enhance the immune response to an antigen associated with the respective archaeosomes/**liposomes**.

SUMM It is another object of this invention to incorporate CoQ_{10} into archaeosomes and conventional **liposomes**, sometimes in combination with polyethylene glycol lipid conjugates, to increase the delivery of various associated compounds to specific organ tissues. . . have been sterically stabilized by association with polyethyleneglycol conjugates, would therefore further increase the utility of archaeosomes and of conventional **liposomes**, for delivery of compounds, including immunogens and CoQ_{10} itself, to phagocytic cells and to specific tissues.

DRWD FIG. 1. Fluorescence micrographs of cells incubated with Methanosarcina mazei archaeosomes, or with conventional **liposomes** prepared from DMPC:DMPG:CHOL, each vesicle type containing CF. Panels A_1, B_1, C_1, murine peritoneal macrophages. Panels A_2, B_2, C_2, J774A.1 macrophages. Panels A_3, B_3, C_3, HEp-2 cells. Panels A_{1-3}, archaeosomes; Panels B_{1-3}, conventional **liposomes**; and panels C_{1-3}, cells without added vesicles. The presence of yellow-fluorescent **liposomes** are indicated in the black and white photos by light areas. Magnification bar=20 μm.

DRWD . . . of murine peritoneal macrophages, J774A.1, and HEp-2 cells

incubated with multilamellar M. mobile archaeosomes (A_1, B_1, C_1), or multilamellar conventional **liposomes** prepared from DMPC:DMPG:CHOL (A_2, B_2, C_2), each vesicle type containing CF. Panels A_{1-2}, J774A.1 cells. Panels B_{1-2}, murine peritoneal macrophages.. . .

DRWD FIG. 3. Tissue distribution of (A) conventional **liposomes** (DSPC:DCP:CHOL) and, (B) CoQ_{10} -conventional **liposomes** (DSPC:DCP:CHOL:Q_{10}) 24 hours after oral and parenteral administration to mice. The data shown are ± sample standard error from the. . .

DRWD FIG. 4. Tissue distribution of (A) PEG-conventional **liposomes** (DSPC:DCP:CHOL:DSPE-PEG) and, (B) PEG-CoQ_{10} -conventional **liposomes** (DSPC:DCP:CHOL:DSPE-PEG:Q_{10}) 24 hours after oral and parenteral administration to mice. The data shown are ± sample standard error from the. . .

DRWD . . . M. smithii (0.57 mg); M. voltae (1.83 mg); M. hungatei (1.09 mg); M. concilii (0.68 mg); M. stadtmanae (0.51 mg); **PC**:PG (DMPC:DMPG, 2.11 mg); **PC**:PG:CHOL (DMPC:DMPG:CHOL, 0.12 mg); and **PC**:DCP:CHOL (DMPC:DCP:CHOL, 0.87 mg). Data are the means from mice in duplicates.

DETD The inventors have discovered that archaeosomes are taken up by phagocytic cells to a greater extent than are conventional **liposomes**. Another aspect of the invention shows the improved uptake by phagocytic cells of both conventional **liposomes** and of archaeosomes, through the incorporation of coenzyme Q_{10} in the respective vesicles. incorporation of CoQ_{10} into conventional **liposomes**, and archaeosomes, also allows for the improved targeting of vesicles to specific tissues in the animal, for vesicles administered via. . . be especially applicable for oral delivery of vaccines. Further, archaeosomes in general, are shown to be superior, compared to conventional **liposomes**, as carriers of antigens, resulting in improved immune response to the antigen administered to an animal such as a human. Further, it is shown that archaeosomes act as superior adjuvants, compared to conventional **liposomes**, resulting in an increased immune response to an antigen administered to an animal such as a human. Also, the duration. . . used in the preparation of vesicles, is shown to improve the immune response to an antigen co-entrapped either in conventional **liposomes** and/or in archaeosomes.

DETD . . . backbone via ether bonds; archaeal or archaeobacterial lipid(s), lipid(s) derived from a member(s) of the class Archaea (synonymous to Archaeobactena); **liposome**, closed vesicle made of lipid bilayer membranes which entrap an aqueous volume, the **liposome** may be **unilamellar** (one bilayer) or multilamellar (multiple bilayers, each separated from the adjoining one by aqueous spaces); conventional **liposome**, a **liposome** made with conventional phospholipids and in some cases including a sterol and may include other compounds that are entrapped within. . . lipids), or a combination of mono and bilayers (if made with diether or other monpolar lipids and tetraether lipids); veside, **liposome** or archaeosome; bare antigen, antigen without adjuvant or vesicle; bare **liposome**/archaeosome, **liposome** or archaeosome without an associated antigen; adjuvant, a substance or material which when administered with an immunogen increases the immune. . .

DETD L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dimyristoylphosphatidylglycerol (DMPG), distearoylphosphatidylcholine (DSPC), dicetylphosphate (DCP, i.e., dihexadecyl phosphate), **cholesterol** (CHOL) (all these were at least 99% pure), 5(6)-carboxyfluorescein (CF), Triton X-100, n-propyl gallate, peroxidase substrate 2,2'-azino-bis (3-ethylbenzthiazoline sulphonic acid),. . .

DETD **Liposome** and Archaeosome Preparation

DETD For **liposome** and archaeosome preparation, the lipids dissolved in chloroform were dried under a stream of N_2, and placed in a lyophilizer. . . consisted of 0.5 ml of 10 mM potassium phosphate buffer, pH 7.14, containing 160 mM NaCl (PBS). Unless indicated

of the hydrated lipids through two stacked 400 nm filters in a LiposoFast apparatus similar to that described by MacDonald et al. (10) to obtain predominantly **unilamellar** vesicles. Archaeobacterial lipids were hydrated for 1-2 hours, or sometimes overnight, at 35° C. and the resulting multilamellar vesicles were. . . ambient temperature. Conventional phospholipids were hydrated (1-2 h) and extruded at 50° C. for DPPC, and at 35° C. for DMPC:DMPG:**cholesterol** (1.8:0.2:1.5, molar ratio) or DMPC:DMPG (1.8:0.2, molar ratio) to obtain **unilamellar** vesicles.

DETD The in vitro uptake of archaeosomes prepared from the TPL extracted from M. mazei and of conventional **liposomes** prepared from DPPC:CHOL (5:5 molar ratio), DSPC:CHOL (5:5 molar ratio) or DSPC:CHOL:DCP (4:5:1 molar ratio) was also studied using cholesteryl. . . required, coenzyme Q_{10} dissolved in chloroform was added (at 5:20 weight/weight ratio of total lipids) before drying the lipids. The **liposomes** and archaeosomes were prepared by the reverse-phase evaporation (REV) method (at 55° C.) combined with bath sonication (at room temperature),. . .

DETD Conventional **liposomes** (DSPC:DCP:CHOL at a molar ratio of 4:1:5), CoQ_{10} -conventional **liposomes** (DSPC:DCP:CHOL:CoQ_{10} at a molar ratio of 3:1:4:2), PEG-conventional **liposomes** (DSPC:DCP:CHOL at a molar ratio of 4:1:5 plus DSPE-PEG at 7% molar ratio of the total lipids), and PEG-CoQ_{10} -conventional **liposomes** (DSPC:DCP:CHOL:CoQ_{10} at a molar ratio of 3:1:4:2 plus DSPE-PEG at 7% molar ratio of the total lipids) were prepared with. . .

DETD . . . archaeosomes (prepared from the TPL, or a lipid obtained in a biologically pure form from the indicated archaeobacterium) and conventional **liposomes** (prepared from DMPC:DMPG at 1.8:0.2 molar ratio, or DMPC:DMPG:CHOL at 1.8:0.2:1.5 molar ratio, or DMPC:DCP:CHOL at 7:1:2 molar ratio) containing. . .

DETD It will be understood by one skilled in the art that the preparation of **liposomes** and archaeosomes of this invention and the association/incorporation of other compounds in the respective vesicles is not limited to the. . .

DETD Peroxidase activity in 5 to 35 μg dry weight of **liposomes** or archaeosomes was assayed in 1 ml reaction mixtures containing 45 mM citric acid, pH 4.0, 2.2 mM H_2O_2,. . .

DETD The amount of protein incorporated into or associated with archaeosomes and **liposomes** to be used for immunizations was quantitated by SDS polyacrylamide gel electrophoresis (PAGE), wherein the lipids separate from the protein. . . The amount of protein incorporated/associated with the vesicles was compared on the basis of salt free dry weights of the **liposomes** or archaeosomes, respectively.

DETD Archaeosomes or **liposomes** containing encapsulated peroxidase were incubated with different cell types to quantitate the respective vesicle binding to mammalian cells. Peritoneal macrophages. . .

DETD Fluorescence microscopy was performed to visualize archaeosome or **liposome** binding to several cell types. For these studies, CF was entrapped within the vesicles at a concentration of only 1.5. . .

DETD These uptake studies were performed essentially as described above, but used **liposomes** and archaeosomes prepared to contain tritiated cholesteryl [1-^{3} H] ether (^{3} H-chol) as a tracer. One micro curie (1 μCi=37. . .

DETD . . . each antigen/adjuvant preparation three mice were injected (unless indicated otherwise) i.p., i.m., or s.c. with antigens encapsulated in archaeosomes or **liposomes** (diluted in sterile PBS, pH 7.1), antigens emulsified in CFA, or with antigens diluted in PBS alone (final volume, 0.2 ml/mouse). For the second immunization, mice were injected with antigens encapsulated in archaeosomes or **liposomes**, or emulsified in IFA, or diluted in PBS. For experiments where a third or fourth injection was required, antigens were. . .

DETD Construction and Characterization of Archaeosomes and **Liposomes**

DETD Peroxidase encapsulated within vesicles was used to quantitate the relative uptake of the various types of archaeosomes and of conventional

liposomes, by eukaryotic cells. The amounts of each vesicle type taken up by adherent cells seeded onto culture wells could be. . .

DETD Archaeosomes were prepared from the TPL extracted from several archaeobacteria, and conventional **liposomes** prepared from two conventional phospholipid formulations that have been frequently used in the prior art (2, 17). The percentage of. . .

DETD . . . the data in FIG. 2, we prepared intermediate-sized vesicles of approximately 200 nm diameter by pressure extrusion of hydrated multilamellar **liposomes** through filters of 400 nm pore size. This resulted in populations of vesicles with the size range distributions defined in Table 1. The distributions were quite narrow except for DPPC **liposomes**, which consisted of two differently sized populations.

DETD Binding of Archaeosomes and Conventional **Liposomes** to Cells

DETD . . . Peroxidase and fluorescence assays were used to quantitate and compare the binding of several types of archaeosomes and of conventional **liposomes** to two phagocytic and three non phagocytic cell lines. The results are illustrated in FIG. 1, and in Tables 2. . . uptake of archaeosomes by the two macrophage lines (murine peritoneal and J774A.1 macrophages) was several times greater than the conventional **liposomes**. Compared with macrophage cell lines, the uptake of archaeosomes and of conventional **liposomes** by non-phagocytic cell lines (Hep-2, HeLa, EJ/28) was substantially lower.

DETD Large, multilamellar vesicles (0.5 to 3 μm) prepared from the polar lipids of M. mazei or from DMPC:DMPG:**cholesterol** gave trends similar to those obtained with the smaller vesicles, i.e. greater uptake of archaeosomes than conventional **liposomes** by murine peritoneal macrophages, and by J774A.1 cells; lesser uptake of both vesicle types by the non-phagocytic HEp-2 cell line. . . .

DETD These data, using encapsulated peroxidase or fluorescent dye as markers, illustrate the enhanced phagocytosis of archaeosomes compared with conventional **liposomes**. The potential advantages for using archaeosomes for delivery of compounds to macrophages is discussed elsewhere in this submission.

DETD . . . inhibition of phagocytosis) of the macrophages over the time periods studied. This indicates that the inhibitors prevented internalization of the **liposomes** into the macrophages. In addition, the time-dependent decline in fluorescence upon readjustment of the temperature of the culture medium from. . .

DETD . . . into archaeosomes prepared from the TPL of M. mazei, which are known to be anionic (19), and into anionic conventional **liposomes** (DSPC:CHOL:DCP), with relatively high entrapment efficiencies (Table 5). However, compared with anionic lipid mixtures, even higher entrapment efficiencies were obtained. . .

DETD The uptake by macrophages, of archaeosomes and of conventional **liposomes** lacking coenzyme Q_{10} is shown as a function of time using ^{3}H-chol as the tracer marker (Table 6A). At. . . indicated times, it can be seen that M. mazei archaeosomes are taken up substantially better than all formulations of conventional **liposomes**, as was also shown in Table 2. The data in Table 6A serve as control values to assess the effect. . . contained coenzyme Q_{10}. This enhancing effect of coenzyme Q_{10} was several times higher with the archaeosomes than with the conventional **liposomes**. Comparable profiles of accumulation and the enhancing effects of coenzyme Q_{10} entrapment on the uptake of archaeosomes and of conventional **liposomes**, was observed at all lipid concentrations tested (compare Tables 7A and 7B).

DETD . . . clearly indicate the potential for inclusion of coenzyme Q_{10} into the vesicles for increased targeting of archaeosomes and of conventional **liposomes** to various cell types. An increased uptake of vesicles containing coenzyme Q_{10} by macrophages (antigen processing cells, and the sites. . . drugs (including antiviral and antimicrobial agents). Another application is to deliver the water-insoluble drug (coenzyme Q_{10}) to mammalian cells, via **liposomes** and archaeosomes.

DETD The tissue distribution profiles of label from conventional **liposomes**

and CoQ_{10} -conventional **liposomes** administered by various routes showed that these were generally similar (FIGS. 3A and 3B). However, the major difference was that in orally administered CoQ_{10} -conventional **liposomes**, no accumulation of the label (at 24 h) was seen in any of the tissues examined, except for in the. . .

DETD . . . reticuloendothelial system (predominantly present in the spleen and liver), and hence prolong the circulation half life in the blood. Such **liposomes** have been called sterically stabilized **liposomes** (24). The tissue distribution profile of PEG-conventional **liposomes** (FIG. 4A) was somewhat similar to that of conventional **liposomes** (FIG. 3A). However, it was surprising to see that when CoQ_{10} was incorporated into the PEG-conventional **liposomes** which were administered orally, there was an increased accumulation of the marker (24 hours post administration) into spleen, liver, intestine,. . . combined accumulation in the spleen and liver was about 8-fold greater. With the i.v. route of administration, the PEG-CoQ_{10} -conventional **liposomes** showed no presence of the marker in the spleen (FIG. 4B).

DETD . . . the intestine and kidneys, the tissue distribution profiles of M. mazei archaeosomes (FIG. 5A) showed differences from those with conventional **liposomes** (FIG. 3A). When M. mazei archaeosomes were administered via i.m., s.c., or i.v. routes, the tissue distribution profile (at 24. . .

DETD . . . the liver and spleen, was about 5-fold better than the highest level obtained (FIG. 4B) with any combination using conventional **liposomes**. For i.m. and i.v. administered PEGCoQ_{10} -archaeosomes, the accumulation of label in the spleen was significantly lower than that obtained. . .

DETD The 48 hour tissue distribution profile of label from orally administered PEG-CoQ_{10} -conventional **liposomes** was similar to that observed at 24 hours (FIG. 4B). However, the 48 hour tissue distribution profile of label from. . .

DETD The data in these figures show that a significant enhancement in the efficacy of the delivery/accumulation of orally administered conventional **liposomes**, to the spleen and liver (the major sites for the antigen processing cells of the immune system), can be achieved. . .

DETD The enhanced uptake of archaeosomes by phagocytic cells, compared to that of conventional **liposomes**, suggested that archaeosomes may be superior as adjuvants and/or carriers of antigens for raising an immune response to an immunogen.. . .

DETD A comparison of the adjuvant/antigen carrier properties of archaeosomes and of conventional **liposomes** was made using BSA as the antigen (FIG. 8). The immune response to BSA was markedly enhanced when it was. . . and the results were again comparable, in some cases, to that achieved with Freund's adjuvant. In contrast, all three conventional **liposome** types yielded substantially lower immunostimulatory effects.

DETD The small adjuvant effect observed with DMPC:DMPG conventional **liposomes** can be improved by the inclusion of increasing amounts of archaeobacterial lipids in the lipids used for vesicle formation. This. . .

DETD Mice were immunized (days 0 and 14), i.p., with conventional **liposomes** (DSPC:DCP:CHOL) containing 15 μg BSA per dose, with and without Q_{10} incorporated (see Table 5 and 6B for compositions). Sera. . . 18 when titred for antibodies (IgG+IgM) had a significantly higher anti-BSA antibody titre (at the 95% confidence limit) when the **liposomes** contained CoQ_{10}.

DETD . . . dialkyl-ether phosphatidylcholines, with BSA as the entrapped antigen, were less efficient in eliciting an immune response in mice compared to **liposomes** made with the diacyl-ester **phosphatidylcholine**. In addition to enhanced adjuvant effects, few boosts are required with archaeosomes. Moreover, the storage stability (shelf life) of archaeosomes. . .

DETD In vitro cytotoxicity studies with several phagocytic and non phagocytic cell lines described earlier indicated that neither archaeosomes nor conventional **liposomes** had any significant adverse effects, as

assessed from cell viability assays, even when the vesicles were tested at lipid concentrations. . .

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DETD TABLE 1

Characteristics of peroxidase-archaeosomes and peroxidase-**liposomes** used in binding assays.[1]

Lipid vesicles	Mean Diameter source for deviation)	(nm ± standard	Peroxidase Activity[2] No Detergent	OGP

M. mazei 189. . . and
Thermoplasma acidophilum.
[2] Activity is expressed as change in absorbency/min/mg dry wt vesicles.
[3] Consisting of two populations of **liposomes** of 324 nm (84%, number weighted) and 2833 nm (16%, number weighted).
DETD TABLE 2

Comparative in vitro uptake (μg/mg cell protein) of archaeosomes and conventional
liposomes by murine peritoneal macrophages and a variety of cell lines.[1]

Lipid source for vesicles	Murine Peritoneal Macrophages	J774A.1 Macrophages	HEp-2 cells. . .

DETD TABLE 3

Relative uptake of archaeosomes and conventional **liposomes** by various cell lines.[1]

Lipid source for vesicles	Mean Diameter (nm ± standard	Vesicles added per assay well	Relative Binding[3] J774A.1	HEp-2	HeLa

. . .
DETD TABLE 4

The effect of inhibitors on the phagocytosis of M. hungatei archaeosomes by J774A.1 macrophages.[1]

Treatment	Relative binding/degradation of **liposomes** by macrophages at different intervals (min)[2] 0	30	60	120	180	240	300	360
None	10	8	5	3	2	1. . .		

DETD TABLE 5

Incorporation of coenzyme Q_{10} into M. mazei archaeosomes and conventional **liposomes**.[1]

Lipid source for vesicles	Starting ratio Q_{10} :lipid (mg:mg)	Loading Entrapment (%)	Ratio (mg Q_{10} /mg lipid)
M. mazei	5:20. . .		

DETD TABLE 6A

Uptake of M. mazei archaeosomes and conventional **liposomes** by J774A.1 macrophages as a function of time, using ^{3}H-chol as the marker.[1]

Lipid source for vesicles	Uptake (cpm)[2] 15. . .

DETD TABLE 6B

Influence of coenzyme Q_{10} on the uptake of M. mazei archaeosomes and conventional **liposomes** by J774A.1 macrophages as a function of time, using ^{3}H-chol as a marker.

Lipid source for vesicles	Uptake (cpm)[2]

DETD TABLE 7A

Uptake of M. mazei archaeosomes and conventional **liposomes** by J774A.1 macrophages as a function of lipid concentration, using ^{3}H-chol as a marker.[1]

Lipid source | Lipid concentration for vesicles | . . . | . . .

DETD TABLE 7B

Influence of coenzyme Q_{10} on the uptake of M. mazei archaeosomes and conventional **liposomes** by J774A.1 macrophages as a function of lipid concentration, using ^{3}H-chol as a marker.[1]

Lipid source | Lipid concentration for vesicles | . . . | . . .

DETD . . . per injection i.p. to each mouse

Immunizations where antigen was not encapsulated consisted of BSA in PBS followed by bare **liposomes** 4 hours later. Bare BSA is a control for antigen alone in PBS. Immunizations were given at 0 and 14. . .

DETD . . . ± 5.9

Archaeosomes	50	186 ± 57	0.86	44.0 ± 11.5
Archaeosomes	10	152 ± 54	1.14	22.4 ± 3.9
Liposomes	0	126 ± 56	1.01	9.8 ± 0.9

[1] Mice (3 per group) were immunized by i.p. injections of various . . .

CLM What is claimed is:

1. A **liposome** composition comprising the total polar lipids extract of an archaeobacterium and an additive selected from the group consisting of coenzyme. . .

2. A **liposome** composition according to claim 1, further comprising a polyethyleneglycol lipid conjugate.

3. A **liposome** composition according to claim 1 comprising the total polar lipids extract of an archaeobacterium and coenzyme Q_{10}.

4. A **liposome** composition according to claim 3, additionally comprising a polyethyleneglycol lipid conjugate.

5. A **liposome** composition according to claim 3, additionally comprising an antigen.

6. A **liposome** composition according to claim 1, wherein the ability to act as an adjuvant or as a carrier for an antigen. . .

7. A **liposome** composition according to claim 1, wherein the archaeobacterium is selected from those which contain tetraether bipolar lipids.

8. A **liposome** composition according to claim 7, wherein the archaeobacterium is selected from the group consisting of Methanospirillun hungatei, Methanosphaera stadtmanae, Methanobrevibacter. . .

9. A **liposome** composition according to claim 1, wherein the **liposome** is **unilamellar**.

10. A **liposome** composition according to claim 1, wherein the **liposome** size is in the range of not less than 50 nm, but less than 500 nm, in diameter.

11. A **liposome** composition according to claim 10, wherein the

liposome size is about 100 to 500 nm in diameter.

12. A **liposome** composition according to claim 1, wherein the archaeobacterium is Halobacterium cutirubrum.

13. A **liposome** composition according to claim 1, wherein the archaeobacterium is Methanosphaera stadtmanae.

14. A **liposome** composition according to claim 1, wherein the archaeobacterium is Methanobrevibacter smithii.

15. A **liposome** composition according to claim 1, wherein the archaeobacterium is Thermoplasma acidophilum.

16. A **liposome** composition according to claim 1, wherein the archaeobacterium is Methanobacterium espanolae.

17. A **liposome** composition according to claim 1, wherein the archaeobacterium is Methanosarcina mazei.

18. A **liposome** composition according to claim 1, comprising the total polar lipids extract of an archaeobacterium and an antigen.

19. A **liposome** composition according to claim 18, wherein the archaeobacterium is Halobacterium cutirubrum.

20. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanosarcina mazei.

21. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanospirillum hungatei.

22. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanococcus jannaschii.

23. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanosphaera stadtmanae.

24. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanobrevibacter smithii.

25. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanococcus voltae.

26. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanobacterium espanolae.

27. A **liposome** composition according to claim 18, wherein the archaeobacterium is Thermoplasma acidophilum.

28. A **liposome** composition according to claim 18, wherein the archaeobacterium is Methanosaeta concilii.

29. A **liposome** composition comprising the total lipids extract of an archaeobacterium and an antigen.

30. A **liposome** composition comprising a lipid selected from the group consisting of 3-O-(3,7,11,15-tetramethyl)hexadecyl-2-O-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-sn-glycero-1-phosphoinositol, 3-O-(3,7,11,15-tetramethyl)hexadecyl-2-O-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-sn-glycero-1-phosphoglycerol, and phosphatidylinositol glycotetraether lipid of mol. wt. 1703. . .

31. A **liposome** composition according to claim 30, wherein the lipid is isolated from an archaeobacterium in a biologically pure form.

32. A **liposome** composition according to claim 30, wherein the

tetramethyl)hexadecyl-sn-glycero-1-phosphoinositol is from Methanosarcina mazei.

33. A **liposome** composition according to claim 30, wherein the 3-O-(3,7,11,15-tetramethyl)hexadecyl-2-O-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-sn-glycero-1-phosphoglycerol is from Methanosarcina mazei.

34. A **liposome** composition according to claim 30, wherein the phosphatidylinositol glycotetraether lipid of mol. wt. 1703 daltons is from Methanobrevibacter smithii.

35. A **liposome** composition comprising 2,3-diphytanyl-sn-glycero-1-phospho-3'-sn-glycero-1'-methylphosphate and an antigen.

36. A **liposome** composition according to claim 35, wherein the 2,3-diphytanyl-sn-glycero-1-phospho-3'-sn-glycero-1'-methylphosphate is from Halobacterium cutirubrum.

37. A **liposome** composition according to claim 35, wherein the lipid is isolated from an archaeobacterium in biologically pure form.

38. A **liposome** composition comprising a conventional phospholipid and 10 to 50%/wt. of the total polar lipids extract of an archacobacterium, additionally comprising. . .

39. A **liposome** composition according to claim 38, wherein the conventional phospholipid is selected from the group consisting of **phosphatidylcholine**, phosphatidylglycerol and mixtures thereof.

40. A **liposome** composition according to claim 38, wherein the total polar lipids extract is from an archaeobacterium having tetraether bipolar lipids.

41. A **liposome** composition, comprising a conventional phospholipid, **cholesterol**, coenzyme Q_{10} and an antigen.

42. A **liposome** composition, consisting essentially of a conventional phospholipid, **cholesterol**, coenzyme Q_{10} and a polyethyleneglycol lipid conjugate.

43. A **liposome** composition according to claim 42, wherein the conventional phospholipid is selected from the group consisting of **phosphatidylcholine**, dicetylphosphate and mixtures thereof.

. . . delivery of an additive selected from an antigen, coenzyme Q_{10} and a mixture thereof to an animal, comprising administering a **liposome** prepared from the total polar lipids extract of an archaeobacterium as a carrier for said additive.

48. A method according to claim 44, wherein the dosage of **liposomes** to be delivered is 4 to 73 mg/kg of animal body weight.

49. A method according to claim 44, wherein the **liposome** is administered to an animal orally, intraperitoneally, intramuscularly, subcutaneously, or intravenously.

50. A method for the delivery of coenzyme Q_{10} and an antigen to an animal, comprising administering a **liposome** prepared from the total polar lipids extract of an archaeobacterium as a carrier for the coenzyme Q_{10} and said antigen.

. . .

51. A method for the delivery of coenzyme Q_{10} and an antigen to an animal, comprising administering a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of

an archaeobacterium and a polyethyleneglycol lipid conjugate; . . .

. . . the immune response to an antigen in a vaccine formulation, comprising administering to an animal a vaccine composition comprising a **liposome** prepared from the total polar lipids extract of an archaeobacterium as an immunestimulating adjuvant for said antigen.

. . . the immune response to an antigen in a vaccine formulation, comprising administering to an animal a vaccine composition comprising a **liposome** prepared from the total polar lipids extract of an archaeobacterium as a carrier for said antigen.

. . . in a vaccine formulation, comprising administering to an animal as a carrier for said antigen a vaccine composition comprising a **liposome** prepared from a polar lipid isolated in a biologically pure form from an archaeobacterium.

56. A method for enhancing the targeted delivery of a **liposome** and coenzyme Q_{10} to specific animal organs, comprising administering a **liposome** prepared from the total polar lipids extract of an archaeobacterium as a carrier for the coenzyme Q_{10}.

57. A method for the delivery of coenzyme Q_{10} to an animal, comprising administering a **liposome** prepared from a composition consisting essentially of the total polar lipids extract of an archaeobacterium, as a carrier for the. . .

58. A method according to claim 57, wherein the **liposome** is delivered to an animal via the oral route.

60. A method for the delivery of coenzyme Q_{10} to an animal, comprising administering to the animal a **liposome** prepared from a composition consisting essentially of CoQ_{10} and the total polar lipids extract of an archaeobacterium and a polyethyleneglycol. . .

61. A method according to claim 60, wherein the **liposome** is administered to an animal via the oral route.

. . . to an antigen in a vaccine formulation, comprising administering to an animal a vaccine composition comprising an antigen and a **liposome** prepared from a composition comprising the total polar lipids extract of an archaeobacterium and a conventional phospholipid as a carrier. . .

. . . enhancing the targeted delivery of coenzyme Q_{10} to specific animal organs and altering the organ tissue distribution profile of a **liposome**, comprising administering to the animal a composition comprising CoQ_{10} and a **liposome** prepared from a conventional phospholipid, **cholesterol** and a polyethyleneglycol lipid conjugate, as a carrier for the coenzyme Q_{10}.

64. A method according to claim 63, wherein the **liposome** is administered to an animal via the oral route.

65. A method for altering tissue distribution of a **liposome** in an animal, by administering to the animal a **liposome** consisting essentially of a conventional phospholipid, **cholesterol** and coenzyme Q_{10}.

66. A method according to claim 65, wherein the **liposome** is administered to the animal via the oral route.

L17 ANSWER 6 OF 15 USPATFULL on STN
2000:53722 Cationic lipids and the use thereof.
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US 6056938 20000502

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Cationic lipid compounds which comprise at least two cationic groups. The cationic lipid compounds are particularly suitable for use as carriers in the intracellular delivery of bioactive agents, including pharmaceuticals and genetic material. Compositions of the present cationic lipid compounds include suspensions, emulsions, micelles and **liposomes**.

AB . . . of bioactive agents, including pharmaceuticals and genetic material. Compositions of the present cationic lipid compounds include suspensions, emulsions, micelles and **liposomes**.

SUMM Various carriers have been developed for use in the transfection of biologically active agents. For example, **liposomes** and polymers have been developed for the transfection of genetic materials, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). However, the currently available carriers, including **liposomes** and polymers, are generally ineffective for the intracellular delivery of biologically active materials in vivo. Moreover, the currently available carriers. . .

SUMM As noted above, **liposomes** have been used as a carrier for the intracellular delivery of biologically active agents, including genetic material. One of the original methods for the use of **liposomes** as carriers for biologically active agents is disclosed in Szoka and Papahadjopoulos, Ann. Rev. Biophysic. Bioeng., Vol. 9, pp. 467-508 (1980). The disclosed method involves the preparation of **liposomes** by the addition of an aqueous solution of genetic material to phospholipids which are dissolved in ether. Evaporation of the. . .

SUMM Another method for encapsulating biologically active agents in **liposomes** involves the extrusion of dehydration-rehydration vesicles. Other methods, in addition to those described above, are known for the encapsulation by **liposomes** of biologically active agents.

SUMM More recently, **liposomes** have been developed from cationic lipids, such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA") or lipids which comprise cationic polymers, for example, polysine. See, e.g., Xiaohuai and Huang, Biochimica et Biophysica Acta, Vol. 1189, pp. 195-203 (1994). **Liposomes** which are prepared from cationic materials (referred to hereinafter as "cationic **liposomes**") have been developed, inter alia, to transfect cells with genetic material, including DNA. It is believed that the cationic **liposomes** bind with the negatively charged phosphate group(s) of the nucleotides in DNA. Studies have shown that cationic **liposomes** mediate transfection of cells with genetic material in vitro more efficiently than other carriers, for example, cationic polymers. In addition, in vitro studies have shown also that cationic **liposomes** provide improved transfection of cells relative to other delivery methods, including electroporation and calcium phosphate precipitation.

SUMM However, the currently available cationic lipids and cationic **liposomes** are generally ineffective for the intracellular delivery of biologically active agents in vivo. Moreover, they are generally ineffective for the. . . it is generally necessary to remove serum from tissue culture baths during gene transfection studies involving cationic lipids and cationic **liposomes**. After transfection, the serum is replaced. This involves additional processing steps which render transfection of cells with cationic lipids and cationic **liposomes** complex and cumbersome.

DETD . . . the mono- or bilayers are generally concentric. The lipid vesicles or vesicular species include such entities commonly referred to as **liposomes**, micelles and the like. Thus, the lipids may be used to form a **unilamellar** vesicle (comprised of one monolayer or bilayer), an oligolamellar vesicle (comprised of about two or about three monolayers or bilayers). . .

DETD **"Liposome"** refers to a generally spherical cluster or aggregate of amphipathic compounds, including lipid compounds, typically in the form of one. . .

DETD . . . below, the cationic lipid compounds are also particularly

suitable for use in the formulation of cationic vesicles, including micelles and **liposomes**. The inventors have found that cationic **liposomes** are also particularly suitable for use as carriers for the intracellular delivery of bioactive agents.

DETD . . . a cationic lipid composition is provided which comprises a cationic vesicular composition. The cationic vesicular composition may comprise micelles and/or **liposomes**. With particular reference to cationic micelle compositions, the following discussion is provided.

DETD As noted above, the cationic vesicular composition may comprise cationic **liposomes**. Cationic **liposomes** are particularly effective as carriers for the intracellular delivery of bioactive agents and are therefore preferred cationic lipid compositions. The present cationic **liposomes** are highly stable and permit substantially complete entrapment of a bioactive agent within the vesicle. Thus, compositions which comprise cationic **liposomes** are highly effective carriers for the transfection of bioactive agents in that the **liposomes** are capable of (A) effectively interacting with the bioactive agent by virtue of electrostatic forces (as discussed above in connection with the cationic lipid compounds, generally); and (B) entrapping the bioactive agent within the **liposome** vesicle. The cationic **liposomes** are also highly biocompatible.

DETD The cationic **liposome** compositions may comprise one or more cationic lipid compounds. In any given **liposome**, the cationic lipid compound(s) may be in the form of a monolayer or bilayer, and the mono- or bilayer lipids. . . one mono- or bilayer, the mono- or bilayers are generally concentric. Thus, the lipids may be used to form a **unilamellar liposome** (comprised of one monolayer or bilayer), an oligolamellar **liposome** (comprised of two or three monolayers or bilayers) or a multilamellar **liposome** (comprised of more than three monolayers or bilayers).

DETD As with the suspensions/emulsions and micelles above, cationic **liposome** compositions are preferably formulated from both the present cationic lipid compounds and additional stabilizing materials, including additional amphipathic compounds. In the case of **liposomes**, the additional amphipathic compounds preferably comprise lipids. A wide variety of additional lipids are available which may be incorporated into the **liposome** compositions. Preferably, the lipids are selected to optimize certain desirable properties of the **liposomes**, including serum stability and plasma half-life. The selection of suitable lipids in the preparation of cationic **liposome** compositions would be apparent to a person skilled in the art and can be achieved without undue experimentation, based on. . .

DETD Lipids which may be used in combination with the present cationic lipid compounds and in the formulation of cationic **liposome** compositions include ZONYL.TM. fluorosurfactants (DuPont Chemicals, Wilmington, Del.) and the fluorine-containing compounds which are described in the following publications: S.. . . are hereby incorporated by reference, in their entireties. Other exemplary lipids which may be used in the preparation of cationic **liposome** compositions include **phosphatidylcholine** with both saturated and unsaturated lipids, including dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine; phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine and dipalmitoylphosphatidylethanolamine (DPPE);. . . (DPPA); palmitic acid; stearic acid; arachidonic acid; oleic acid; fatty acids; lipids with ether and ester-linked fatty acids; polymerizable lipids; **cholesterol**, **cholesterol** sulfate and **cholesterol** hemisuccinate; 12-{[(7'-diethylaminocoumarin-3-yl)carbonyl]methylamino}octadecanoic acid; N-[12-{[(7'-diethylaminocoumarin-3-yl)carbonyl]methylamino}-octadecanoyl]-2-aminopalmitic acid; cholesteryl-4'-trimethylaminobutanoate; N-succinyldioleoylphosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinyl-glycerol; 1-hexadecyl-2-palmitoylglycerophosphatidylethanolamine; and palmitoylhomocysteine.

DETD Lipids bearing polymers, including the hydrophilic polymers poly(ethylene glycol) (PEG), polyvinylpyrrolidone, and poly(vinyl

present invention. Examples of suitable hydrophilic polymers include, for example, PEG 2,000, PEG 5,000 and PEG 8,000,. . . based on the present disclosure. Polymers which may be incorporated via alkylation or acylation reactions onto the surface of the **liposome** are particularly useful for improving the stability and size distribution of the **liposomes**. Exemplary lipids which bear hydrophilic polymers include, for example, dipalmitoylphosphatidylethanolamine-PEG, dioleoylphosphatidylethanolamine-PEG and distearylphosphatidylethanolamine-PEG.

DETD Other materials for use in the preparation of cationic **liposome** compositions, in addition to those exemplified above, would be apparent to one skilled in the art based on the present. . .

DETD A wide variety of methods are available in connection with the preparation of cationic **liposome** compositions. Accordingly, the cationic **liposomes** may be prepared using any one of a variety of conventional **liposome** preparatory techniques which will be apparent to those skilled in the art. These techniques include solvent dialysis, French press, extrusion (with or without freeze thaw), reverse phase evaporation, microemulsification and simple freeze-thawing. The **liposomes** may also be prepared by various processes which involve shaking or vortexing. This may be achieved, for example, by the. . .

DETD Additional methods for the preparation of **liposome** compositions from the cationic lipid compounds of the present invention include, for example, sonication, chelate dialysis, homogenization, solvent infusion, spontaneous formation, solvent vaporization, controlled detergent dialysis, and others, each involving the preparation of **liposomes** in various fashions. Methods which involve freeze-thaw techniques are preferred in connection with the preparation of **liposomes** from the cationic lipid compounds of the present invention. Suitable freeze-thaw techniques are described, for example, in copending U.S. application. . . 07/838,504, filed Feb. 19, 1992, the disclosures of which are incorporated herein by reference in their entirety. Preparation of the **liposomes** may be carried out in a solution, such as an aqueous saline solution, aqueous phosphate buffer solution, or sterile water, containing one or more bioactive agents, so that the bioactive agent is encapsulated in the **liposome** or incorporated into the **liposome** membrane. Alternatively, the bioactive agents may be added to previously formed **liposomes**.

DETD The size of the **liposomes** can be adjusted, if desired, by a variety of techniques, including extrusion, filtration, sonication and homogenization. In addition, the size of the **liposomes** can be adjusted by the introduction of a laminar stream of a core of liquid into an immiscible sheath of liquid. Other methods for adjusting the size of the cationic **liposomes** and for modulating the resultant liposomal biodistribution and clearance of the **liposomes** would be apparent to one skilled in the art based on the present disclosure. Preferably, the size of the cationic **liposomes** is adjusted by extrusion under pressure through pores of a defined size. Although **liposomes** employed in the subject invention may be of any one of a variety of sizes, preferably the **liposomes** are small, that is, less than about 100 nanometer (nm) in outside diameter.

DETD . . . 47-55 (1987); International Application Serial No. PCT/US89/05040; U.S. Pat. No. 4,162,282; U.S. Pat. No. 4,310,505; U.S. Pat. No. 4,921,706; and **Liposome** Technology, Gregoriadis, G., ed., Vol. I, pp. 29-31, 51-67 and 79-108 (CRC Press Inc., Boca Raton, Fla. 1984), the disclosures. . .

DETD Although any of a number of varying techniques can be used, the **liposomes** of the present invention are preferably prepared using a shaking technique. Preferably, the shaking techniques involve agitation with a mechanical. . .

DETD . . . that the gaseous substances and/or precursors thereto are incorporated in compositions which are suspensions/emulsions or vesicular compositions, including micelles and **liposomes**. Incorporation of the gaseous substances and/or precursors thereto in the cationic lipid compositions may be achieved by using any of. . .

DETD ... In preferred embodiments, the gaseous substances and/or gaseous precursor materials are incorporated in vesicular compositions, with micelles and **liposomes** being preferred. **Liposomes** are particularly preferred because of their high stability and biocompatability. As discussed in detail below, vesicles in which a gas. . .

DETD . . . or in the lipid membranes. Thus, in certain embodiments, the bioactive agent may be coated on the surface of the **liposomes** or micelles and/or in the lipid membranes, in addition to, or instead of, being entrapped within the vesicles.

DETD . . . lipoprotein (HDL) receptor for the treatment of liver disease; thymidine kinase for the treatment of ovarian cancer, brain tumors, or **human immunodeficiency virus** (**HIV**) infection; HLA-B7 for the treatment of malignant melanoma; IL-2 for the treatment of neuroblastoma, malignant melanoma or kidney cancer; interleukin-4 (IL-4) for the treatment of cancer; **HIV** env for the treatment of **HIV** infection; antisense ras/p53 for the treatment of lung cancer; and Factor VIII for the treatment of Hemophilia B. Such therapies. . .

DETD . . . with one or more bioactive agents. The cationic lipid compositions may comprise cationic suspensions/emulsions and/or cationic vesicular compositions, including cationic **liposome** compositions and/or cationic micelle compositions. In addition, the cationic lipid compositions can comprise one or more cationic lipid compounds optionally. . . In the case of vesicular compositions, it is contemplated that the bioactive agent is entrapped within the vesicle of the **liposome** or micelles. In certain cases, the bioactive agent can be incorporated also into the membrane walls of the vesicle. In. . . case, the bioactive agent is then added to the lipid composition prior to use. For example, an aqueous mixture of **liposomes** and gas may be prepared to which the bioactive agent is added and which is agitated to provide the cationic **liposome** formulation. The cationic **liposome** formulation is readily isolated also in that the gas- and/or bioactive agent-filled **liposome** vesicle generally float to the top of the aqueous solution. Excess bioactive agent can be recovered from the remaining aqueous. . .

DETD . . . culture applications, the cationic lipid formulations can be added to the cells in cultures and then incubated. If desired, where **liposomes** are employed, energy, such as sonic energy, may be applied to the culture media to burst the **liposomes** and release any therapeutic agents.

DETD . . . compound used, the therapeutic or diagnostic use contemplated, and the form of the formulation, for example, suspension, emulsion, micelle or **liposome**, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased. . .

DETD . . . As one skilled in the art would recognize, the lipid formulations, including those which comprise suspensions/emulsions and vesicles, such as **liposomes** and micelles, may be coated with certain materials to minimize uptake by the reticuloendothelial system. Suitable coatings include, for example,. . .

DETD LIPOFECTAMINE.TM. and LIPOFECTIN® were purchased from Gibco BRL, a division of Life Technologies, Inc. (Gaithersburg, Md.). LIPOFECTAMINE.TM. is a 3:1 **liposome** formulation of N-[2-({2,5-bis(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate and dioleoylphosphatidylethanolamine ("DOPE"). LIPOFECTIN® is a **liposome** formulation of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA") and DOPE. (See Proc. Natl. Acad. Sci. USA, Vol. 84, p. 7413 (1987).) TRANSFECTAM.TM. was. . .

CLM What is claimed is:

49. A cationic lipid composition according to claim 1 which is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

57. A lipid formulation according to claim 55 which is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

58. A lipid formulation according to claim 57 wherein said bioactive

agent is substantially entrapped within said micelles or **liposomes**.

68. A process according to claim 65 wherein said composition is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

69. A process according to claim 68 comprising substantially entrapping said bioactive agent within said micelles or **liposomes**.

75. A method of claim 73 wherein said composition is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

76. A method of claim 75 wherein said bioactive agent is substantially entrapped within said micelles or **liposomes**.

88. A cationic vesicle composition according to claim 87 wherein said vesicles are selected from the group consisting of **unilamellar** vesicles, oligolamellar vesicles and multilamellar vesicles.

91. A cationic vesicle composition according to claim 88 wherein said vesicles comprise **unilamellar** vesicles.

109. A cationic lipid composition according to claim 85 wherein said lipids comprise **unilamellar** lipids, oligolamellar lipids or multilamellar lipids.

112. A cationic lipid composition according to claim 109 wherein said lipids comprise **unilamellar** lipids.

131. A cationic vesicle according to claim 130 which is selected from the group consisting of **unilamellar** vesicles, oligolamellar vesicles and multilamellar vesicles.

132. A cationic vesicle according to claim 131 which comprises **unilamellar** vesicles.

L17 ANSWER 7 OF 15 USPATFULL on STN
1999:159514 Stabilized compositions of fluorinated amphiphiles for methods of therapeutic delivery.
Unger, Evan C., Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5997898 19991207
APPLICATION: US 1995-465868 19950606 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB Stabilized compositions comprising, in combination with a gas, a fluorinated amphiphilic compound. The compositions are particularly suitable for use in diagnostic applications, including ultrasound. The compositions can take the form of vesicular compositions, such as micelles and **liposomes**.
AB . . . for use in diagnostic applications, including ultrasound. The compositions can take the form of vesicular compositions, such as micelles and **liposomes**.
SUMM . . . walls surrounding an internal void that is filled with a gas or a precursor thereto. Exemplary bubbles include, for example, **liposomes**, micelles and the like. As discussed more fully hereinafter, the effectiveness of bubbles as contrast agents depends upon various factors,. . .
SUMM . . . the prior art is generally inadequate for use as contrast agents. For example, the prior art discloses bubbles, including gas-filled **liposomes**, which comprise lipoidal walls or membranes. See, e.g., Ryan et al., U.S. Pat. Nos. 4,900,540 and 4,544,545; Tickner et al.,. . .
SUMM . . . hydrophilic portion and a water-insoluble, hydrophobic portion. Preferred amphiphilic compounds are characterized by a polar head group, for example, a **phosphatidylcholine** group, and one or more nonpolar,

aliphatic chains, for example, palmitoyl groups. Fluorinated amphiphile" refers to an amphiphilic compound in. . .

SUMM . . . or bilayer, the mono- or bilayers are generally concentric. The vesicles described herein include such entities commonly referred to as **liposomes**, micelles, bubbles, microbubbles, microspheres and the like. Thus, the amphiphilic compounds may be used to form a **unilamellar** vesicle (comprised of one monolayer or bilayer), an oligolamellar vesicle (comprised of about two or about three monolayers or bilayers). . .

SUMM "**Liposome**" refers to a generally spherical cluster or aggregate of amphiphilic compounds, including lipid compounds, typically in the form of one. . .

SUMM . . . lipids, and especially phospholipids, which comprise a polar head group including, for example, a phosphorylated head group, such as a **phosphatidylcholine** group, or a sulfated head group, and at least one nonpolar aliphatic chain, such as a palmitoyl group. In such. . .

SUMM . . . aggregated, for example, substantially randomly, as well as compositions in which the fluorinated amphiphilic compounds form vesicles, including micelles and **liposomes**. Incorporation of the gases and/or gaseous precursors in the present compositions may be achieved by using any of a number. . .

SUMM In preferred embodiments, the gases and/or gaseous precursor materials are incorporated in vesicular compositions, with micelles and **liposomes** being preferred. As discussed in detail below, vesicles in which a gas or gas precursor or both are encapsulated are. . .

SUMM In certain preferred embodiments, the additional amphiphilic materials comprise a lipid compound. Suitable lipids include, for example, phospholipids, such as **phosphatidylcholine** with both saturated and unsaturated fatty acids, including dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine; phosphatidylethanolamines, such as dipalmitoylphosphatidylethanolamine, dioleoylphosphatidylethanolamine, N-succinyldioleoylphosphatidylethanolamine and. . . such as dipalmitolylphosphatidic acid; palmitic acid; stearic acid; arachidonic acid; oleic acid; lipids bearing polymers, such as polyethyleneglycol or polyvinylpyrrolidone; **cholesterol** and **cholesterol** hemisuccinate; 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; N-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid; cholesteryl-(4'-trimethylamino)butanoate; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinylglycerol; and palmitoylhomocysteine.

SUMM . . . materials include, for example, oils, such as peanut oil, canola oil, olive oil, safflower oil and corn oil; lecithin; sphingomyelin; **cholesterol** and derivatives thereof; squalene; terpenes and terpenoid compounds; triglycerides; gums, such as xanthan, tragacanth, locust bean, guar and carrageenan gums;. . .

SUMM . . . certain preferred embodiments of the invention, the stabilized compositions comprise a vesicular composition. The vesicular compositions may comprise micelles and/or **liposomes**. A wide variety of methods are available for the preparation of vesicular compositions, including, for example, shaking, drying, gas-installation, spray. . .

SUMM The vesicular composition may also comprise **liposomes**. In any given **liposome**, the involved amphiphilic compound(s), including fluorinated and nonfluorinated amphiphilic compound, may be in the form of a monolayer or bilayer,. . . one mono- or bilayer, the mono- or bilayers are generally concentric. Thus, the amphiphilic compounds may be used to form **unilamellar liposomes** (comprised of one monolayer or bilayer), oligolamellar **liposomes** (comprised of two or three monolayers or bilayers) or multilamellar **liposomes** (comprised of more than three monolayers or bilayers).

SUMM A wide variety of methods are available in connection with the preparation of **liposome** compositions. Accordingly, the **liposomes** may be prepared using any one of a variety of conventional liposomal preparatory techniques which will be apparent to those. . . These techniques include solvent dialysis, French press, extrusion (with or without freeze-thaw), reverse phase evaporation, microemulsification and

simple freeze shaking. The **liposomes** may also be prepared by various processes which involve shaking or vortexing. This may be achieved, for example, by the. . .

SUMM Additional methods for the preparation of **liposome** compositions include, for example, sonication, chelate dialysis, homogenization, solvent infusion, spontaneous formation, solvent vaporization, controlled detergent dialysis, and others, each involving the preparation of **liposomes** in various fashions. Methods which involve freeze-thaw techniques are preferred in connection with the preparation of **liposomes**. Suitable freeze-thaw techniques are described, for example, in copending U.S. application Ser. No. 07/838,504, filed Feb. 19, 1992, the disclosures of which are incorporated herein by reference in their entirety. Preparation of the **liposomes** may be carried out in a solution, such as an aqueous saline solution, aqueous phosphate buffer solution, or sterile water.

SUMM As noted above, the size of the vesicles, including **liposomes**, can be adjusted, if desired, by a variety of techniques, including extrusion, filtration, sonication and homogenization. In addition, the size of the **liposomes** can be adjusted by the introduction of a laminar stream of a core of liquid into an immiscible sheath of liquid. Other methods for adjusting the size of the **liposomes** and for modulating the resultant liposomal biodistribution and clearance of the **liposomes** would be apparent to one skilled in the art based on the present disclosure. Preferably, the size of the **liposomes** is adjusted by extrusion under pressure through pores of a defined size. Although **liposomes** employed in the subject invention may be of any one of a variety of sizes, the **liposomes** are preferably small, that is, less than about 100 nanometer (nm) in outside diameter.

SUMM . . . 47-55 (1987); International Application Serial No. PCT/US89/05040; U.S. Pat. No. 4,162,282; U.S. Pat. No. 4,310,505; U.S. Pat. No. 4,921,706; and **Liposome** Technology, Gregoriadis, G., ed., Vol. I, pp. 29-31, 51-67 and 79-108 (CRC Press Inc., Boca Raton, Fla. 1984), the disclosures. . .

SUMM In the case of vesicular compositions, including micelles and **liposomes**, it is contemplated that the bioactive agent is preferably entrapped within a void of the vesicles. In certain cases, the. . .

SUMM . . . lipoprotein (HDL) receptor for the treatment of liver disease; thymidine kinase for the treatment of ovarian cancer, brain tumors, or **human immunodeficiency virus** (**HIV**) infection; HLA-B7 for the treatment of malignant melanoma; IL-2 for the treatment of neuroblastoma, malignant melanoma or kidney cancer; interleukin-4 (IL-4) for the treatment of cancer; **HIV** env for the treatment of **HIV** infection; antisense ras/p53 for the treatment of lung cancer; and Factor VIII for the treatment of Hemophilia B. Such therapies. . .

SUMM . . . case, stabilized compositions are prepared as described above in the presence of a bioactive agent. Thus, for example, micelles and **liposomes** can be prepared in the presence of a bioactive agent. The preparation can involve, for example, bubbling a gas directly. . . case, the bioactive agent is then added to the amphiphilic composition prior to use. For example, an aqueous mixture of **liposomes** and gas may be prepared to which the bioactive agent is added and which is agitated to provide the **liposome** formulation. The **liposome** formulation is readily isolated also in that the gas- and/or bioactive agent-filled **liposome** vesicles generally float to the top of the aqueous solution. Excess bioactive agent can be recovered from the remaining aqueous. . .

SUMM . . . cell culture applications, the amphiphilic formulations can be added to the cells in cultures and then incubated. If desired, where **liposomes** are employed, energy, such as sonic energy, may be applied to the culture media to burst the **liposomes** and release any therapeutic agents.

SUMM . . . art would recognize, based on the present disclosure, the formulations, including those which comprise suspensions, emulsions and/or vesicles, such as **liposomes** and micelles, may be coated with certain materials to minimize uptake by the reticuloendothelial system. Suitable coatings include, for example,. . .

SUMM . . . a bioactive agent, and diagnostic or therapeutic use contemplated, and the form of the involved compositions, for example, micelle or **liposome**, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased. . .

DETD . . . for 5 minutes at 4,200 r.p.m. on a Wig-L-Bug.TM. (Crescent Dental, Lyons, Ill.) shaking apparatus to provide perfluoropropane gas-filled vesicles (**liposomes**) having a mean diameter of about 3 μm. The gas-filled vesicles will be stable for several weeks at room temperature.. . .

DETD . . . reequilibrated to ambient pressure over a period of 48 hours. The resulting vesicular composition will comprise gas (nitrogen) filled vesicles (**liposomes**) having a mean diameter of 200 nm. The vesicles can be stored dry until use, as desired, and can be. . .

DETD . . . (Crescent Dental, Lyons, Ill.) shaking apparatus while the temperature of the vial is maintained at 35° C. Perfluoropentane gas-filled vesicles (**liposomes**) will be obtained.

DETD . . . that the perfluoropentane gas is replaced with a mixture of perfluoropentane gas and nitrogen gas (1:1 v/v). Cooling the resulting **liposomes** to below 30° C. may result in the condensation of the perfluoropentane gas. However, the condensed perfluoropentane will exist as. . .

DETD . . . (Crescent Dental, Lyons, Ill.) shaking apparatus while the temperature of the vial is maintained at 60° C. Perfluorohexane gas-filled vesicles (**liposomes**) will be obtained.

CLM What is claimed is:

. . . wherein said gas is sulfur hexafluoride and is encapsulated in said vesicles which are selected from the group consisting of **liposomes**, micelles and microspheres, and wherein said fluorinated amphiphilic compound is of formula (II): ##STR7## wherein: m is 0 to about. . . 4. A method according to claim 1 wherein said vesicles are selected from the group consisting of micelles and **liposomes**.

5. A method according to claim 1 wherein said vesicles comprise **unilamellar** vesicles.

L17 ANSWER 8 OF 15 USPATFULL on STN

1999:65064 Transdermal delivery system for antigen.

Alving, Carl R., Bethesda, MD, United States
Glenn, Gregory M., Bethesda, MD, United States
The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 5910306 19990608
APPLICATION: US 1996-749164 19961114 (8)
DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A transdermal **liposome** system delivers antigen to immune cells without perforation of the skin, and induces an immune response in an animal or human. The system uses **liposomes** to deliver a variety of antigens which can elicit an antigen-specific immune response (e.g., humoral and/or cellular effectors) after topical application of a formulation containing **liposomes** and antigen to intact skin of the animal or human.

AB A transdermal **liposome** system delivers antigen to immune cells without perforation of the skin, and induces an immune response in an animal or human. The system uses **liposomes** to deliver a variety of antigens which can elicit an antigen-specific immune response (e.g., humoral and/or cellular effectors) after topical application of a formulation containing **liposomes** and antigen to intact skin of the animal or human.

SUMM The invention relates to transdermal delivery of antigen by a **liposome** formulation to induce an antigen-specific immune response.

SUMM **Liposomes** are smectic mesophases, which have been defined in the following manner by D. M. Small (Handbook of Lipid Research, Vol.. . .

SUMM . . . of antigen, Paul et al. (1995) and Paul and Cevc (1995) (hereinafter "the Cevc lab") were not able to use **liposomes** for transdermal immunization. The Cevc lab used three different lipid

formulations: mixed micelles, **liposomes**, and transferosomes in attempting to cause the transdermal delivery of antigen. Lipid was provided as an ethanol solution of soybean **phosphatidylcholine** (SPC); **liposomes** were formed by sonication, then freeze-thawed, and finally filtered for the purposes of sterilization and improved sample homogeneity. Mixed micelles. . . salt (BS) in a mole ratio of 1:1, transferosomes contained SPC and BS in a mole ratio of 9:2, and **liposomes** contained SPC but no BS.

SUMM Because they contain a significant proportion of bile salts, mixed micelles and transferosomes cannot be considered **liposomes** (i.e., smectic mesophases) as stated by D. M. Small (Handbook of Lipid Research, Vol. 4, Plenum, N.Y., p. 95): "Class. . . Na cholate, Na deoxycholate, and Na chenodeoxycholate), saponins, and rosin soaps." As noted above in Small's definition of smectic mesophases, **liposomes** are a type of liquid crystals.

SUMM . . . measured by titer of antigen-specific antibody. Topically applied formulations of antigen in solution, antigen and mixed micelles, and antigen and **liposomes** (i.e., smectic mesophases) did not induce an immune response equivalent to that induced by subcutaneous injection. Therefore, there was a positive control (i.e., antigen and transfersomes) to validate their negative conclusion that a formulation of antigen and **liposomes** did not cause transdermal immunization.

SUMM Despite the aforementioned contrary teaching, we have found that **liposomes** do provide a transdermal delivery system for antigen that can induce an antigen-specific immune response.

SUMM In one embodiment of the invention, a formulation containing **liposomes** and antigen is applied to intact skin of an organism, the antigen is presented to immune cells, and an antigen-specific. . .

SUMM . . . third embodiment of the invention, a patch for use in the above methods is provided. The patch comprises a dressing, **liposomes**, and a therapeutically effective amount of antigen. The dressing may be occlusive or non-occlusive. The patch may include additional antigens. . .

SUMM **Liposomes** may be multilamellar, paucilamellar, or **unilamellar**; the **liposomes** may be phospholipid **liposomes** containing phospholipid, sterol, or a mixture thereof. The phospholipid may be **phosphatidylcholine**, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidic acid, lysophosphatide, sphingomyelin, or mixtures thereof. The sterol is a derivative based on the cyclopentanophenanthrene nucleus, and is preferably **cholesterol**, **cholesterol** esters, **cholesterol** sulphates, or mixtures thereof. A **liposome** may also contain a nonphospholipid such as, for example, ceramide, cerebroside, glycosphingolipid, sphingolipid, free fatty acids, eicosanoids, and lipid vitamins. **Liposomes** may contain a nonionic amphiphile such as, for example, polyoxyethylene fatty acid ester, polyoxyethylene fatty acid ether, diethanolamide, long chain. . . glyceryl ester, polyoxyethylene glyceryl diester, glycerol stearate, glycerol distearate, glycerol oleate, glycerol dioleate, glycerol palmitate, glycerol dipalmitate, or mixtures thereof. **Liposomes** may contain an ionic amphiphile such as, for example, betaine, sarcosinate, monomeric alkyd, dimeric alkyd, dimethyl distearyl amine, or mixtures. . .

SUMM **Liposomes** of the invention are closed vesicles surrounding an internal aqueous space. The internal compartment is separated from the external medium. . . surfactant, synthetic or natural lipid, saturated or unsaturated lipid, and charged or neutral lipid, either with or without a sterol. **Liposomes** may be either multilamellar, paucilamellar, or **unilamellar**, and may be made in different sizes: small being less than 25 nm, intermediate being 25 nm to 500 nm, and large being greater than 500 nm. A typical **liposome** is composed of dimyristoyl **phosphatidylcholine** (DMPC), dimyristoyl phosphatidylglycerol (DMPG), and **cholesterol**, with or without lipid A, in a multilamellar configuration, and has a population of sizes from about 0.2 μm to. . .

SUMM **Liposomes** of the invention are used as a transdermal delivery system of agents that induce an immune response. These agents as. . .

SUMM **liposomes** may be preformed and then mixed with antigen. **Liposomes** may also be formed so as to contain antigen inserted in the lipid bilayer, in the inner aqueous spaces, associated. . . in any combination of these arrangements. The antigen may be dissolved or suspended, and then added to (a) the preformed **liposomes** in a lyophilized state, (b) dried lipids as a swelling solution or suspension, or (c) the solution of lipids used to form **liposomes**. The **liposomes** may either be used unwashed, or washed prior to use to remove antigen that is not associated with the **liposome**.

SUMM The **liposomes** may contain a single antigen, more than one antigen, or the **liposomes** containing separate antigens may be mixed into a single **liposome** formulation. The multivalent antigen formulation may be used to induce an immune response to more than one antigen at the. . .

SUMM The **liposomes** may be applied in the form of an emulsion, gel, solution, suspension, or other forms known in the art.

SUMM . . . antigens can be delivered to the immune system, especially specialized immune cells underlying the skin. A mixture of antigen and **liposomes**; or antigen encapsulated in, attached to, or associated with the lipid bilayer of **liposomes** may be applied with or without adjuvants.

SUMM . . . bound to any particular theory but only to provide an explanation for our observations, it is presumed that the transdermal **liposome** delivery system carries antigen to cells of the immune system where an immune response is induced. The antigen may pass. . . macrophage, Langerhans cell, dendritic cell, B lymphocyte, or Kupffer cell) that presents processed antigen to a T lymphocyte. Passage of **liposomes** through the stratum corneum may not be necessary to deliver antigen to cells of the immune system.

SUMM **LIPOSOME** LIPID

SUMM **Liposomes** may be prepared using a variety of techniques and membrane lipids (reviewed in Gregoriadis, 1993).

SUMM Natural sources may provide **liposome** lipid such as, for example, lecithin (i.e., **phosphatidylcholine**): egg yolk, soybean, and brain. Synthetic lipids are preferred for their chemical purity. Synthetic lecithins are available with fatty acyl. . .

SUMM **Liposomes** of the invention may be formed from a phospholipid such as, for example, **phosphatidylcholine**, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylserine, phosphatidic acid, phosphatidylinositol, lysophosphatide, and sphingomyelin. If a sterol is used to stabilize the lipid bilayer, it is preferably **cholesterol**, a **cholesterol** ester, or a **cholesterol** sulphate. **Cholesterol** may be recrystallized to avoid the possibility of immunosuppression or toxicity due to oxidation products.

SUMM **Liposomes** of the invention may be formed from lipids extracted from the stratum corneum including, for example, ceramide and **cholesterol** derivatives (Wertz, 1992).

SUMM **Liposomes** of the invention may contain a nonionic amphiphile as a major structural component (by weight) of the lipid bilayer. The. . . diester, glycerol stearate, glycerol distearate, glycerol oleate, glycerol dioleate, glycerol palmitate, glycerol dipalmitate, or mixtures thereof. Nonionic amphiphiles that form **liposomes** in the presence of steroid are disclosed in U.S. Pat. No. 4,917,951, incorporated herein by reference. The lipid bilayer may. . .

SUMM Stability, rigidity, and permeability of the **liposome** is altered by changes in lipid composition. Membrane fluidity is generally controlled by the fatty acyl chains of the lipid.. . .

SUMM Antigen is solubilized prior to mixing with **liposomes**. Suitable buffers include, but are not limited to, phosphate buffered saline Ca++ /Mg++ free (PBS), normal saline, and TRIS buffer.. . .

SUMM Antigen can also be solubilized in a detergent (e.g., a cell membrane extract) along with the lipids themselves, and **liposomes** are then formed by removal of the detergent by dilution, dialysis, or column chromatography. Certain antigens such as, for example,. . . those from a virus (e.g., hepatitis A) need not be soluble per se, but can be incorporated directly into a **liposome** in the form of a virosome.

SUMM . . . Shafara et al., 1995; Smedila et al., 1994; U.S. Pat. Nos. 5,314,808 and 5,436,126), herpes simplex virus 1 or 2, **human**

immunodeficiency virus (Lopez et al., 1990), influenza, Norwalk, papilloma virus, parvovirus B19, rabies, rotavirus, rubella, rubeola, varicella, and yellow fever. Parasites include,. . .

SUMM The formulation of **liposomes** and antigen may also contain an adjuvant. Adjuvants are substances that are used to specifically or nonspecifically potentiate an antigen-specific. . .

SUMM . . . derived from the lipopolysaccharide (LPS) of gram-negative bacterial endotoxin. It is an outstanding adjuvant that can be incorporated into the **liposome** bilayer to induce an immune response to a **liposome**-associated antigen (Alving, 1993). Lipid A is actually a heterogeneous mixture of compounds having similar structures (Banerji and Alving, 1979). The. . .

SUMM PREPARATION OF **LIPOSOMES**

SUMM . . . upon storage. Therefore, at one- to three-month intervals, chloroform is redistilled prior to its use as the solvent in forming **liposomes**. After distillation, 0.7% ethanol can be added as a preservative. Ethanol and methanol are other suitable solvents.

SUMM The lipid solution used to form **liposomes** is placed in a round-bottomed flask. Pear-shaped boiling flasks are preferred, particularly those flasks sold by Lurex Scientific (Vineland, N.J.,. . . volume of the flask should be more than ten times greater than the volume of the anticipated aqueous suspension of **liposomes** to allow for proper agitation during **liposome** formation.

SUMM To encapsulate antigen into **liposomes**, an aqueous solution containing antigen may be added to lyophilized **liposome** lipids in a volume that results in a concentration of approximately 200 mM with respect to **liposome** lipid, and shaken or vortexed until all the dried **liposome** lipids are wet. The **liposome**-antigen mixture may then be incubated for 18 hours to 72 hours at 40° C. The **liposome**-antigen formulation may be used immediately or stored for several years.

SUMM It may be advantageous to employ the **liposome**-antigen mixture directly in the transdermal delivery system. But if removal of non-encapsulated antigen from the mixture is desired, approximately 20 volumes of buffer may be added to the mixture and the **liposomes** pelleted by centrifugation at 25,000 g to 30,000 g for 30 minutes at 20° C. to 25° C. After removal of the clear (or slightly turbid) supernatant fraction, the **liposome** pellet may be suspended to the desired final volume (10 mM to 200 mM with respect to **liposome** lipids) with the appropriate buffer.

SUMM Alternatively, **liposomes** may be formed as described above but without addition of antigen to the aqueous solution. Antigen may then be added to the preformed **liposomes** and, therefore, antigen would be in solution and/or associated with, but not encapsulated by, the **liposomes**.

SUMM A method for forming **liposomes** containing at least two lipids or amphiphiles is disclosed in U.S. Pat. No. 5,260,065, incorporated herein by reference.

SUMM A method and apparatus for forming **liposomes** without using a solvent are disclosed in U.S. Pat. Nos. 4,895,452 and 4,911,928, incorporated herein by reference. The lipid phase. . . the formulation is heated until flowing, and then blended with an excess of the aqueous phase under shear conditions until **liposomes** are formed. If an oil or a water immiscible component of the formulation (e.g., antigen or adjuvant) is to be encapsulated in the **liposome** or incorporated in the lipid bilayer, such a component can be blended first with the lipid phase before hydration by. . .

SUMM Other methods for forming **liposomes** are disclosed in U.S. Pat. Nos. 4,089,801, 4,196,191, 4,235,871, 4,485,054, 4,508,703, 4,731,210, 4,897,269, 4,963,297, 4,975,282, 5,008,050, 5,059,421, and 5,169,637, incorporated herein by reference. A method for obtaining an oil-in-water emulsion containing **liposomes** is disclosed in U.S. Pat. No. 3,957,971, incorporated herein by reference; a method for obtaining a water-in-oil emulsion containing **liposomes** is disclosed in U.S. Pat. No. 5,256,422, incorporated herein by reference. If the process for forming **liposomes** would denature the antigen in the formulation, the antigen will be mixed with the formed **liposomes**. Therefore, antigen would not be encapsulated by **liposomes** formed by such an antigen-denaturing process

but, instead, the antigen would only be mixed in solution and/or associated with the **liposomes**.

SUMM Lipid compositions and methods for forming **unilamellar liposomes** are disclosed in U.S. Pat. Nos. 4,853,228 and 5,008,050, incorporated herein by reference.

SUMM Several types of **liposomes**, such as **unilamellar**, paucilamellar, or multilamellar vesicles, might be used as a transdermal delivery system for antigens. However, because they are easier to. . .

SUMM **Liposomes** have been used as carriers in adjuvants to enhance the immune response to antigens mixed with, encapsulated in, attached to, or associated with **liposomes**. For previous vaccine applications using **liposomes**, the formulation was injected through the skin with a needles, as are the majority of licensed vaccines. Injection of vaccines. . .

SUMM The transdermal **liposome** system may be applied directly to the skin and allowed to air dry, held in place with a dressing or. . . held by a device such as a stocking or shirt or sprayed onto the skin to maximize contact of the **liposomes** with the skin. The formulation may be applied in an absorbant dressing or gauze. The formulation may be covered with. . .

SUMM . . . the immunizing antigen, such as cholera toxin. These antigen-specific antibodies are induced when antigen is delivered through the skin by **liposomes**.

SUMM Alving et al. (1986) injected **liposomes** comprising lipid A as an adjuvant for inducing an immune response to cholera toxin (CT) in rabbits and to a. . . immune response to cholera toxin or to the synthetic malaria protein was markedly enhanced by encapsulating the antigen within the **liposomes** containing lipid A, compared to similar **liposomes** lacking lipid A. Several antigens derived either from the circumsporozoite protein (CSP) or from merozoite surface proteins of Plasmodium falcipacrum have been encapsulated in **liposomes** containing lipid A. All of the malaria antigens that have been encapsulated in **liposomes** containing lipid A have been shown to induce humoral effectors (i.e., antigen-specific antibodies), and some have been shown to induce. . .

DETD As an example of the present invention applied to transdermal delivery of the antigen cholera toxin, multilamellar **liposomes** containing dimyristoyl **phosphatidylcholine**, dimyristoyl phosphatidylglycerol, **cholesterol** and lipid A (prepared according to Alving et al., 1993; Alving et al., 1995; Richards et al., 1995, incorporated herein. . .

DETD Dimyristoyl **phosphatidylcholine** (DMPC), dimyristoyl phosphatidylglycerol (DMPG), and **cholesterol** (Chol) were obtained from Avanti Polar Lipids (Alabaster, Ala.). Lipid A (primarily monophosphoryl) and cholera toxin (CT) were obtained from. . .

DETD . . . minutes. The lipids were further dried under low vacuum (less than 50 torr) for 1 hour in a dessicator. The **liposomes** were formed by removing the lipid film from the wall of the flask with 20 ml of swelling solution (sterile. . .

DETD These **liposomes** were mixed in their lyophilized state with cholera toxin (CT) at 5 mg/ml in normal saline (0.154 M NaCl, pH. . .

DETD A total of 100 μl of the **liposome**-antigen formulation was applied to 4 cm^2 of shaved skin on the back of each BALB/c mouse. Approximately 25 μl of. . .

DETD Boosting immunization was repeated using the same **liposome**-antigen formulation and technique three weeks after primary immunization.

DETD Table 1. Antibody response to cholera toxin. BALB/c mice immunized with cholera toxin in **liposomes** containing lipid A, applied on the skin. Anti-cholera toxin antibodies measured using ELISA, reported in OD units on individual mice.. . .

DETD Lyophilized lipids were prepared, and the **liposomes** were formed in the presence of cholera toxin antigen as in Example 1. The formulation containing cholera toxin was used for transdermal immunization unwashed, or washed by adding approximately 20 volumes of buffer and pelleting the **liposomes** by centrifugation at 25,000 g to 30,000 g for 20 minutes at 20° C. to 25° C. After removal of the clear (or slightly turbid) supernatant fraction, the **liposome** pellet was suspended to a

final concentration of 500 μg/ml cholera toxin as determined by a modified Lowry assay.

DETD Mice in the unwashed **liposome** group were immunized transdermally with 100 μl of **liposome**-antigen formulation (with or without lipid A) as in Example 1; mice in the washed **liposome** group were immunized transdermally with 500 μl of **liposome**-antigen formulation (with or without lipid A) as in Example 1. The amount of cholera toxin used in the above cases. . .

DETD . . . areas for two hours. The oral immunization groups were fed the equivalent of 25 μg of cholera toxin of the **liposome**-antigen formulation used for transdermal immunization.

DETD . . . each group. OD units represent the serum dilution at which the optical density is equal to 1. Abbreviations: L is **liposome**, LA is lipid A, and CT is cholera toxin.

DETD The antigen-specific immune response associated with the groups of unwashed **liposome**-antigen formulation applied transdermally was measured to demonstrate the kinetics and maintenance of the immune response (Table 3) and the difference in IgG subclasses induced by **liposome**-antigen formulations with or without lipid A (Table 4).

DETD . . . each group. OD units represent the serum dilution at which the optical density is equal to 1. Abbreviations: L is **liposome**, LA is lipid A, and CT is cholera toxin.

DETD . . . in Glenn et al. (1995) and reported as the geometric mean and standard error of the mean. Abbreviations: L is **liposome**, LA is lipid A, and CT is cholera toxin.

DETD . . . et al., 1995) may be used as an antigen in the transdermal delivery system described above. Transdermal application of the **liposome**-antigen formulation may be used to vaccinate against dengue-2 viral infection and assessed in either animal or human trials.

DETD Multiple dengue envelope proteins from serotypes 1-4 may be used in transdermal immunization: simultaneously, in succession, in the same **liposome**, in separate **liposomes**, with or without adjuvants as described previously. Induction of an antigen-specific immune response may be assayed as described above for. . .

DETD Alving, C. R. et al. (1986) Effectiveness of **liposomes** as potential carriers of vaccines: Applications to cholera toxin and human malaria sporozoite antigen. Vaccine, 4:166-172.

DETD Alving, C. R. (1991) **Liposomes** as carriers of antigens and adjuvants. J. Immunol. Meth., 140:1-13.

DETD Alving, C. R. (1993) Lipopolysaccharide, lipid A, and **liposomes** containing lipid A as immunologic adjuvants. Immunobiology, 187:430-436.

DETD Alving, C. R. et al. (1993) The preparation and use of **liposomes** in immunological studies. In: **Liposome** Technology, vol. 3, (Ed., Gregoriadis, G.), CRC Press, Boca Raton, pp. 317-343.

DETD Alving, C. R. et al. (1995) **Liposomes** as carriers of peptide antigens: Induction of antibodies and cytotoxic T lymphocytes to conjugated and unconjugated peptides. Immunol. Rev., 145:5-31.

DETD Banerji, B. and Alving, C. R. (1979) Lipid A from endbtoxin: Antigenic activities of purified fractions in **liposomes**. J. Immunol., 123:2558-2562.

DETD Glenn, G. M. et al. (1995) Murine IgG subclass antibodies to antigens incorporated in **liposomes** containing lipid A. Immunol. Lett., 47:73-78.

DETD Gregoriadis, G. (1993) **Liposome** Preparation and Related Techniques, 2nd Ed., CRC Press, Boca Raton.

DETD Richards, R. L. et al. (1988) **Liposomes**, lipid A, and aluminum hydroxide enhance the immune response to a synthetic malaria sporozoite antigen. Infect. Immun., 56:682-686.

DETD Wertz, P. W. (1992) **Liposome** dramatics: Chemicals aspects of the skin lipid approach. In: **Liposome** Dramatics (Eds., Braun-Falco, O. et al.), Springer-Verlag, New York, pp. 38-43.

DETD White, K. et al. (1993) Induction of cytolytic and antibody responses using Plasmodium falciparum repeatless circumsporozoite protein encapsulated in **liposomes**. Vaccine, 11:1341-1346.

CLM What is claimed is:

. . . immune response to an antigen comprising: (a) applying a formulation to intact skin of an organism, wherein the formulation comprises

liposomes and the antigen, and (b) inducing the immune response in the organism without perforating the skin, wherein the immune response. . .

19. The method of claim 1, wherein the **liposomes** are **unilamellar**.

20. The method of claim 1, wherein the **liposomes** are paucilamellar, or multilamellar.

21. The method of claim 1, wherein the **liposomes** are formed from phospholipid, or a mixture of phospholipid and sterol.

22. The method of claim 21, wherein the phospholipid is selected from the group consisting of **phosphatidylcholine**, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylserine, phosphatidic acid, phosphatidylinositol, lysophosphatide, sphingomyelin, and mixtures thereof.

23. The method of claim 21, wherein the sterol comprises **cholesterol**.

24. The method of claim 1, wherein the **liposomes** are formed from a nonionic amphiphile.

26. The method of claim 1, wherein the **liposomes** are formed from an ionic amphiphile.

L17 ANSWER 9 OF 15 USPATFULL on STN
1999:30374 Immunostimulating and immunopotentiating reconstituted influenza virosomes and vaccines containing them.
Gluck, Reinhard, Spiegel/Bern, Switzerland
Mischler, Robert, Worblaufen, Switzerland
Schweiz, Serum- & Impfinstitut Bern, Bern, Switzerland (non-U.S. corporation)
US 5879685 19990309
APPLICATION: US 1994-225740 19940411 (8)
PRIORITY: EP 1991-107527 19910508
EP 1991-107647 19910510
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Immunostimulating reconstituted influenza virosomes (IRIVs) are provided wherein an antigen or a combination of antigens are incorporated into a virosome further containing a mixture of phospholipids, an essentially reconstituted functional virus envelope, and influenza hemagglutinin protein (HA). The HA induces fusion of the IRIV with cellular membranes and thereby induces lysis of the IRIV after its endocytosis by antigen presenting cells.

SUMM . . . including the self aggregation characteristic of antigens, such as the soluble antigen of hepatitis B virus. In the case of **liposomes** or oily droplets, there is a combined effect of particulateness and slow absorption, such as with alum precipitation.

SUMM . . . Slow release is favored by adsorbing antigens onto aluminum hydroxide ("alum precipitation"); placing antigens into water-in-oil emulsions; incorporating antigens into **liposomes**; and other similar manipulations. This method is conceptually close to the one described in section A.

SUMM . . . proinflammatory and encephalopathogenic potential. Surface active agents display a number of side reactions: they are irritating, proinflammatory, they bind to **cholesterol** and lyse cells. Interleukins can provoke systemic reactions and, therefore, routine use in mass vaccination may be undesirable.

SUMM . . . the antigen particulate often goes in parallel with a significant loss of the amount of antigen. The immunostimulatory effect of **liposome**-associated antigen on the humoral response is a widely recognized phenomenon, but immunopotentiation is limited and the mechanism by which this. . .

DETD . . . phospholipids or a mixture thereof. At least it contains two

different compounds selected from the group of glycero phospholipids, such as **phosphatidylcholine** or phosphatidylethanolamine, and **cholesterol**. **Phosphatidylcholine** and phosphatidylethanolamine are preferred, in particular in a ratio of 4:1. In preferred embodiments of the present invention, the ratio. . .

DETD . . . influenza virus envelope's membrane part. In a preferred embodiment the essentially reconstituted functional virus envelopes exhibit the form of a **unilamellar** bilayer. An example of such a lacking component is the matrix protein of the natural influenza virus envelope.

DETD . . . bacteria or parasites, or a toxin. Examples of viruses are hepatitis A, B, C, D or E virus, Polio virus, **HIV**, Rabies virus, Influenza virus or Parainfluenza virus. Examples of bacteria are Pseudomonas, Kiebsiella, E. coli Salmonella typhi, Haemophilus influenzae, Bordetelia. . .

DETD (A) A dispersion of **phosphatidylcholine** (e.g., lecithin, SIGMA) (75%), phosphatidylethanolamine (SIGMA) (20%) and **cholesterol** (SIGMA) (5%) (all phospholipids 1-2% (w/v)=0.013-0.027M) in 0.1M NaCl containing 0.01M Tris/HCl, pH 7.3 was prepared by mixing these compounds. . .

DETD . . . ultrasonification apparatus (Bransonic, Branson Europe BV, frequency 50 kHz ±10%). 10 seconds of ultrasonic shocks repeated every minute yielded small **unilamellar** IRIVs. The sample volumes and column dimensions were such that a complete separation of IRIVs eluted at the void volume. . .

DETD **Phosphatidylcholine** (**PC**; Sigma Chemical Co., St. Louis, Mo.) and phosphatidylethanolamine (PE; Sigma) (75%:25% wt/wt) were suspended in 0.01M Tris - 0.1M NaCl,. . .

DETD . . . compared with live influenza virus in fusion activity with model membranes. FIG. 8 shows the kinetics of fluorescence de-quenching with DOPC-**cholesterol liposomes**. The increase in fluorescence is expressed in % florescence de-quenching (FDQ), calculated according to Luscher and Gluck.

CLM What is claimed is:

. . . 21 amino, acid residue segment of the HA_2 using octaethyleneglycol mono (n-dodecyl) ether (OEG) detergent; b. dispersing a glycerophospholipid and **cholesterol** in an aqueous solution so as to obtain an **unilamellar liposome**; c. combining the mixture and the **liposome** so as to obtain an **unilamellar** immunostimulating reconstituted influenza virosome (IRIV) and; d. adsorbing or attaching onto the surface of the IRIV a combination of immunostimulating. . .
2. The method of claim 1 wherein the glycerophospholipid is **phosphatidylcholine** or phosphatidylethanolamine.

3. The method of claim 1 wherein the glycerophospholipid is a mixture of **phosphatidylcholine** and phosphatidylethanolamine in a ratio of about 1:1 to about 20:1.

. . . 5 wherein the viruses are selected from the group consisting of Hepatitis A, B, C, D or E, polio virus, **HIV**, rabies virus, influenza virus, and parainfluenza virus.

. . . 21 amino acid residue segment of the HA_2 using octaethyleneglycol mono (n-dodecyl) ether (OEG) detergent; b. dispersing a glycerophospholipid and **cholesterol** in an aqueous solution so as to obtain an **unilamellar liposome**; c. combining the mixture and the **liposome** so as to obtain an **unilamellar** IRIV having an essentially reconstituted viral envelope; and d. adsorbing or attaching onto the surface of the IRIV an immunostimulating. . .
12. The method of claim 10 wherein the glycerophospholipid is **phosphatidylcholine** or phosphatidylethanolanine.

13. The method of claim 10 wherein the glycerophospholipid is a mixture of **phosphatidylcholine** and phosphatidylethanolamine in a ratio of about 1:1 to about 20:1.

. . . claim 15 wherein the virus is selected from the group consisting of

virus, and parainfluenza virus.

19. An IRIV of claim 15 wherein the virus is **HIV**.

L17 ANSWER 10 OF 15 USPATFULL on STN
1998:134594 Cationic lipids and the use thereof.
Unger, Evan C., Tucson, AZ, United States
Shen, Dekang, Tucson, AZ, United States
Wu, Guanli, Tucson, AZ, United States
ImaRx Pharmaceutical Corp., Tucson, AZ, United States (U.S. corporation)
US 5830430 19981103
APPLICATION: US 1995-391938 19950221 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Cationic lipid compounds which comprise at least two cationic groups. The cationic lipid compounds are particularly suitable for use as carriers in the intracellular delivery of bioactive agents, including pharmaceuticals and genetic material. Compositions of the present cationic lipid compounds include suspensions, emulsions, micelles and **liposomes**.

AB . . . of bioactive agents, including pharmaceuticals and genetic material. Compositions of the present cationic lipid compounds include suspensions, emulsions, micelles and **liposomes**.

SUMM Various carriers have been developed for use in the transfection of biologically active agents. For example, **liposomes** and polymers have been developed for the transfection of genetic materials, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). However, the currently available carriers, including **liposomes** and polymers, are generally ineffective for the intracellular delivery of biologically active materials in vivo. Moreover, the currently available carriers. . .

SUMM As noted above, **liposomes** have been used as a carrier for the intracellular delivery of biologically active agents, including genetic material. One of the original methods for the use of **liposomes** as carriers for biologically active agents is disclosed in Szoka and Papahadjopoulos, Ann. Rev. Biophysic. Bioeng., Vol. 9, pp. 467-508 (1980). The disclosed method involves the preparation of **liposomes** by the addition of an aqueous solution of genetic material to phospholipids which are dissolved in ether. Evaporation of the. . .

SUMM Another method for encapsulating biologically active agents in **liposomes** involves the extrusion of dehydration-rehydration vesicles. Other methods, in addition to those described above, are known for the encapsulation by **liposomes** of biologically active agents.

SUMM More recently, **liposomes** have been developed from cationic lipids, such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA") or lipids which comprise cationic polymers, for example, polysine. See, e.g., Xiaohuai and Huang, Biochimica et Biophysica Acta, Vol. 1189, pp. 195-203 (1994). **Liposomes** which are prepared from cationic materials (referred to hereinafter as "cationic **liposomes**") have been developed, inter alia, to transfect cells with genetic material, including DNA. It is believed that the cationic **liposomes** bind with the negatively charged phosphate group(s) of the nucleotides in DNA. Studies have shown that cationic **liposomes** mediate transfection of cells with genetic material in vitro more efficiently than other carriers, for example, cationic polymers. In addition, in vitro studies have shown also that cationic **liposomes** provide improved transfection of cells relative to other delivery methods, including electroporation and calcium phosphate precipitation.

SUMM However, the currently available cationic lipids and cationic **liposomes** are generally ineffective for the intracellular delivery of biologically active agents in vivo. Moreover, they are generally ineffective for the. . . it is generally necessary to remove serum from tissue culture baths during gene transfection studies involving cationic lipids and cationic **liposomes**. After transfection, the serum

is replaced. This involves additional processing steps which render transfection of cells with cationic lipids and cationic **liposomes** complex and cumbersome.

DETD . . . bilayer, the mono- or bilayers are generally,concentric. The lipid vesicles or vesicular species include such entities commonly referred to as **liposomes**, micelles and the like. Thus, the lipids may be used to form a **unilamellar** vesicle (comprised of one monolayer or bilayer), an oligolamellar vesicle (comprised of about two or about three monolayers or bilayers). . .

DETD **"Liposome"** refers to a generally spherical cluster or aggregate of amphipathic compounds, including lipid compounds, typically in the form of one. . .

DETD . . . below, the cationic lipid compounds are also particularly suitable for use in the formulation of cationic vesicles, including micelles and **liposomes**. The inventors have found that cationic **liposomes** are also particularly suitable for use as carriers for the intracellular delivery of bioactive agents.

DETD . . . a cationic lipid composition is provided which comprises a cationic vesicular composition. The cationic vesicular composition may comprise micelles and/or **liposomes**. With particular reference to cationic micelle compositions, the following discussion is provided.

DETD As noted above, the cationic vesicular composition may comprise cationic **liposomes**. Cationic **liposomes** are particularly effective as carriers for the intracellular delivery of bioactive agents and are therefore preferred cationic lipid compositions. The present cationic **liposomes** are highly stable and permit substantially complete entrapment of a bioactive agent within the vesicle. Thus, compositions which comprise cationic **liposomes** are highly effective carriers for the transfection of bioactive agents in that the **liposomes** are capable of (A) effectively interacting with the bioactive agent by virtue of electrostatic forces (as discussed above in connection with the cationic lipid compounds, generally); and (B) entrapping the bioactive agent within the **liposome** vesicle. The cationic **liposomes** are also highly biocompatible.

DETD The cationic **liposome** compositions may comprise one or more cationic lipid compounds. In any given **liposome**, the cationic lipid compound(s) may be in the form of a monolayer or bilayer, and the mono- or bilayer lipids. . . one mono- or bilayer, the mono- or bilayers are generally concentric. Thus, the lipids may be used to form a **unilamellar liposome** (comprised of one monolayer or bilayer), an oligolamellar **liposome** (comprised of two or three monolayers or bilayers) or a multilamellar **liposome** (comprised of more than three monolayers or bilayers).

DETD As with the suspensions/emulsions and micelles above, cationic **liposome** compositions are preferably formulated from both the present cationic lipid compounds and additional stabilizing materials, including additional amphipathic compounds. In the case of **liposomes**, the additional amphipathic compounds preferably comprise lipids. A wide variety of additional lipids are available which may be incorporated into the **liposome** compositions. Preferably, the lipids are selected to optimize certain desirable properties of the **liposomes**, including serum stability and plasma half-life. The selection of suitable lipids in the preparation of cationic **liposome** compositions would be apparent to a person skilled in the art and can be achieved without undue experimentation, based on. . .

DETD Lipids which may be used in combination with the resent cationic lipid compounds and in the formulation of cationic **liposome** compositions include ZONYL.TM. fluoro surfactants (DuPont Chemicals, Wilmington, Del.) and the fluorine-containing compounds which are described in the following publications:. . . are hereby incorporated by reference, in their entireties. Other exemplary lipids which may be used in the preparation of cationic **liposome** compositions include **phosphatidylcholine** with both saturated and unsaturated lipids, including dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine; phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine and

dipalmitoylphosphatidylethanolamine (DPPE), . . . (DPPC), palmitic acid; stearic acid; arachidonic acid; oleic acid; fatty acids; lipids with ether and ester-linked fatty acids; polymerizable lipids; **cholesterol**, **cholesterol** sulfate and **cholesterol** hemisuccinate; 12-{[(7'-diethylaminocoumarin-3-yl)carbonyl]methylamino}octadecanoic acid; N-[12-{[(7'-diethylaminocoumarin-3-yl)carbonyl]methylamino}-octadecanoyl]-2-aminopalmitic acid; cholesteryl-4'-trimethylaminobutanoate; N-succinyldioleoylphosphatidyl-ethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinyl-glycerol; 1-hexadecyl-2-palmitoylglycerophosphatidylethanolamine; and palmitoylhomocysteine.

DETD Lipids bearing polymers, including the hydrophilic polymers poly(ethylene glycol) (PEG), polyvinylpyrrolidone, and poly(vinyl alcohol), may also be included in the **liposome** compositions of the present invention. Examples of suitable hydrophilic polymers include, for example, PEG 2,000, PEG 5,000 and PEG 8,000,. . . based on the present disclosure. Polymers which may be incorporated via alkylation or acylation reactions onto the surface of the **liposome** are particularly useful for improving the stability and size distribution of the **liposomes**. Exemplary lipids which bear hydrophilic polymers include, for example, dipalmitoylphosphatidylethanolamine-PEG, dioleoylphosphatidylethanolamine-PEG and distearylphosphatidyl-ethanolamine-PEG.

DETD Other materials for use in the preparation of cationic **liposome** compositions, in addition to those exemplified above, would be apparent to one skilled in the art based on the present. . .

DETD A wide variety of methods are available in connection with the preparation of cationic **liposome** compositions. Accordingly, the cationic **liposomes** may be prepared using any one of a variety of conventional **liposome** preparatory techniques which will be apparent to those skilled in the art. These techniques include solvent dialysis, French press, extrusion (with or without freeze thaw), reverse phase evaporation, microemulsification and simple freeze-thawing. The **liposomes** may also be prepared by various processes which involve shaking or vortexing. This may be achieved, for example, by the. . .

DETD Additional methods for the preparation of **liposome** ompositions from the cationic lipid compounds of the resent invention include, for example, sonication, chelate dialysis, homogenization, solvent infusion, spontaneous formation, solvent vaporization, controlled detergent dialysis, and others, each involving the preparation of **liposomes** in various fashions. Methods which involve freeze-thaw techniques are preferred in connection with the preparation of **liposomes** from the cationic lipid compounds of the present invention. Suitable freeze-thaw techniques are described, for example, in copending U.S. application. . . abandoned, filed Feb. 19, 1992, the disclosures of which are incorporated herein by reference in their entirety. Preparation of the **liposomes** may be carried out in a solution, such as an aqueous saline solution, aqueous phosphate buffer solution, or sterile water, containing one or more bioactive agents, so that the bioactive agent is encapsulated in the **liposome** or incorporated into the **liposome** membrane. Alternatively, the bioactive agents may be added to previously formed **liposomes**.

DETD The size of the **liposomes** can be adjusted, if desired, by a variety of techniques, including extrusion, filtration, sonication and homogenization. In addition, the size of the **liposomes** can be adjusted by the introduction of a laminar stream of a core of liquid into an immiscible sheath of liquid. Other methods for adjusting the size of the cationic **liposomes** and for modulating the resultant liposomal biodistribution and clearance of the **liposomes** would be apparent to one skilled in the art based on the present disclosure. Preferably, the size of the cationic **liposomes** is adjusted by extrusion under pressure through pores of a defined size. Although **liposomes** employed in the subject invention may be of any one of a variety of sizes, preferably the **liposomes** are small, that is, less than about 100 nanometer (nm) in outside diameter.

DETD . . . 47-55 (1987); International Application Serial No.

Pat. No. 4,921,706; and **Liposome** Technology, Gregoriadis, G., ed., Vol. I, pp. 29-31, 51-67 and 79-108 (CRC Press Inc., Boca Raton, Fla. 1984), the disclosures. . .

DETD Although any of a number of varying techniques can be used, the **liposomes** of the present invention are preferably prepared using a shaking technique. Preferably, the shaking techniques involve agitation with a mechanical. . .

DETD . . . that the gaseous substances and/or precursors thereto are incorporated in compositions which are suspensions/emulsions or vesicular compositions, including micelles and **liposomes**. Incorporation of the gaseous substances and/or precursors thereto in the cationic lipid compositions may be achieved by using any of. . .

DETD In preferred embodiments, the gaseous substances and/or gaseous precursor materials are incorporated in vesicular compositions, with micelles and **liposomes** being preferred. **Liposomes** are particularly preferred because of their high stability and biocompatability. As discussed in detail below, vesicles in which a gas. . .

DETD . . . or in the lipid membranes. Thus, in certain embodiments, the bioactive agent may be coated on the surface of the **liposomes** or micelles and/or in the lipid membranes, in addition to, or instead of, being entrapped within the vesicles.

DETD . . . lipoprotein (HDL) receptor for the treatment of liver disease; thymidine kinase for the treatment of ovarian cancer, brain tumors, or **human immunodeficiency virus** (**HIV**) infection; HLA-B7 for the treatment of malignant melanoma; IL-2 for the treatment of neuroblastoma, malignant melanoma or kidney cancer; interleukin-4 (IL-4) for the treatment of cancer; **HIV** env for the treatment of **HIV** infection; antisense ras/p53 for the treatment of lung cancer; and Factor VIII for the treatment of Hemophilia B. Such therapies. . .

DETD . . . with one or more bioactive agents. The cationic lipid compositions may comprise cationic suspensions/emulsions and/or cationic vesicular compositions, including cationic **liposome** compositions and/or cationic micelle compositions. In addition, the cationic lipid compositions can comprise one or more cationic lipid compounds optionally. . . In the case of vesicular compositions, it is contemplated that the bioactive agent is entrapped within the vesicle of the **liposome** or micelles. In certain cases, the bioactive agent can be incorporated also into the membrane walls of the vesicle. In. . . case, the bioactive agent is then added to the lipid composition prior to use. For example, an aqueous mixture of **liposomes** and gas may be prepared to which the bioactive agent is added and which is agitated to provide the cationic **liposome** formulation. The cationic **liposome** formulation is readily isolated also in that the gas- and/or bioactive agent-filled **liposome** vesicle generally float to the top of the aqueous solution. Excess bioactive agent can be recovered from the remaining aqueous. . .

DETD . . . culture applications, the cationic lipid formulations can be added to the cells in cultures and then incubated. If desired, where **liposomes** are employed, energy, such as sonic energy, may be applied to the culture media to burst the **liposomes** and release any therapeutic agents.

DETD . . . compound used, the therapeutic or diagnostic use contemplated, and the form of the formulation, for example, suspension, emulsion, micelle or **liposome**, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased. . .

DETD . . . As one skilled in the art would recognize, the lipid formulations, including those which comprise suspensions/emulsions and vesicles, such as **liposomes** and micelles, may be coated with certain materials to minimize uptake by the reticuloendothelial system. Suitable coatings include, for example,. . .

DETD LIPOFECTAMINE.TM. and LIPOFECTIN® were purchased from Gibco BRL, a division of Life Technologies, Inc. (Gaithersburg, Md.). LIPOFECTAMINE.TM. is a 3:1 **liposome** formulation of N-[2-({2,5-bis(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl-N,N-dimethyl-

dioleoylphosphatidylethanolamine ("DOPE"). LIPOFECTIN® is a **liposome** formulation of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA") and DOPE. (See Proc. Natl. Acad. Sci. USA, Vol. 84, p. 7413 (1987).) TRANSFECTAM.TM. was. . .

CLM What is claimed is:

51. The cationic lipid composition according to claim 49 which is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

61. The cationic vesicle composition according to claim 60 wherein said vesicles are selected from the group consisting of **unilamellar** vesicles, oligolamellar vesicles and multilamellar vesicles.

64. The cationic vesicle composition according to claim 61 wherein said vesicles comprise **unilamellar** vesicles.

82. The cationic lipid composition according to claim 58 wherein said lipids comprise **unilamellar** lipids, oligolamellar lipids or multilamellar lipids.

85. The cationic lipid composition according to claim 82 wherein said lipids comprise **unilamellar** lipids.

105. The lipid formulation according to claim 103 which is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

106. The lipid formulation according to claim 105 comprising said bioactive agent entrapped within said micelles or **liposomes**.

113. The process according to claim 110 wherein said composition is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

114. The process according to claim 113 comprising entrapping said bioactive agent within said micelles or **liposomes**.

120. The method of claim 118 wherein said composition is selected from the group consisting of micelles, **liposomes** and mixtures thereof.

121. The method of claim 120 comprising said bioactive agent entrapped within said micelles or **liposomes**.

128. The cationic vesicle according to claim 127 which is selected from the group consisting of **unilamellar** vesicles, oligolamellar vesicles and multilamellar vesicles.

129. The cationic vesicle according to claim 128 which comprises **unilamellar** vesicles.

L17 ANSWER 11 OF 15 USPATFULL on STN

1998:72264 Therapeutic drug delivery systems.

Unger, Evan C., Tucson, AZ, United States
Fritz, Thomas A., Tucson, AZ, United States
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US 5770222 19980623

APPLICATION: US 1995-472305 19950607 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Therapeutic drug delivery systems comprising gas-filled microspheres comprising a therapeutic are described. Methods for employing such

microspheres in therapeutic drug delivery applications are also provided. Drug delivery systems comprising gas-filled **liposomes** having encapsulated therein a drug are preferred. Methods of and apparatus for preparing such **liposomes** and methods for employing such **liposomes** in drug delivery applications are also disclosed.

AB . . . are described. Methods for employing such microspheres in therapeutic drug delivery applications are also provided. Drug delivery systems comprising gas-filled **liposomes** having encapsulated therein a drug are preferred. Methods of and apparatus for preparing such **liposomes** and methods for employing such **liposomes** in drug delivery applications are also disclosed.

SUMM . . . cells. These mechanisms include techniques such as calcium phosphate precipitation and electroporation, and carriers such as cationic polymers and aqueous-filled **liposomes**. These methods have all been relatively ineffective in vivo and only of limited use for cell culture transfection. None of. . .

SUMM Conventional, liquid-containing **liposomes** have been used to deliver genetic material to cells in cell culture but have generally been ineffective in vivo for cellular delivery of genetic material. For example, cationic **liposome** transfection techniques have not worked effectively in vivo. More effective means are needed to improve the cellular delivery of therapeutics. . .

SUMM In addition, the present invention provides methods and apparatus for preparing gas-filled **liposomes** suitable for use as drug delivery agents. Preferred methods of the present invention provide the advantages, for example, of simplicity. . .

SUMM The gas-filled **liposomes** are particularly useful as drug carriers. Unlike **liposomes** of the prior art that have a liquid interior suitable only for encapsulating drugs that are water soluble, the gas-filled **liposomes** made according to the present invention are particularly useful for encapsulating lipophilic drugs. Furthermore, lipophilic derivatives of drugs may be. . .

DRWD FIG. 1 is a diagrammatical representation of a gas-filled **liposome** having a therapeutic compound embedded within the wall of a **liposome** microsphere, and the subsequent release of the therapeutic upon the application of ultrasound.

DRWD FIG. 2 is a diagrammatical depiction of a gas-filled **liposome** having a therapeutic compound embedded within the inner layer of the wall of a **liposome** microsphere, and exposed to the gas-filled interior, and the subsequent release of the therapeutic upon the application of ultrasound.

DRWD FIG. 3 is a diagrammatical illustration of a gas-filled **liposome** having a therapeutic compound embedded within the outer layer of the wall of a **liposome** microsphere, and exposed to the gas-filled interior, and the subsequent release of the therapeutic upon the application of ultrasound.

DRWD FIG. 4 is a diagrammatical representation of a gas-filled **liposome** microsphere having a therapeutic compound embedded within the inner and outer layers of the wall of a **liposome** microsphere, and exposed to both the internal gas-filled void, and the exterior environment, and the subsequent release of the therapeutic. . .

DRWD FIG. 5 is a diagrammatical depiction of a gas-filled **liposome** microsphere having a therapeutic compound attached to the interior of the **liposome**, and the subsequent release of the therapeutic upon the application of ultrasound.

DRWD FIG. 6 is a diagrammatical depiction of a gas-filled **liposome** microsphere having a therapeutic compound attached to the exterior of a **liposome** microsphere, and the subsequent release of the therapeutic upon the application of ultrasound.

DRWD FIG. 7 is a diagrammatical illustration of a gas-filled **liposome** microsphere having a therapeutic compound, such as a negatively charged drug (A) or a positively charged drug (B) attached to the interior and the exterior of a **liposome** microsphere, and the subsequent release of the therapeutic upon the application of ultrasound.

DRWD FIG. 8 is a diagrammatical illustration of a gas-filled **liposome** microsphere having a therapeutic compound encapsulated within the

upon the. . .

DRWD . . . is a view, partially schematic, of a preferred apparatus according to the present invention for preparing the therapeutic containing gas-filled **liposome** microspheres of the present invention.

DRWD FIG. 10 shows a preferred apparatus for filtering and/or dispensing therapeutic containing gas-filled **liposome** microspheres of the present invention.

DRWD FIG. 11 depicts a preferred apparatus for filtering and/or dispensing therapeutic containing gas-filled **liposome** microspheres of the present invention.

DRWD FIG. 13 is a graphical representation of the dB reflectivity of gas-filled **liposomes** substantially devoid of liquid in the interior thereof prepared by the vacuum drying gas instillation method, without any drugs encapsulated. . .

DRWD FIG. 14 shows a preferred apparatus for preparing the drug containing vacuum dried gas instilled **liposomes**, and the drug containing gas-filled **liposomes** substantially devoid of liquid in the interior thereof prepared by the vacuum drying gas instillation method.

DRWD FIGS. 15A and B are micrograph which shows the sizes of gas-filled **liposomes** of the invention before (A) and after (B) filtration.

DRWD FIGS. 16A and B are graphically depicts the size distribution of gas-filled **liposomes** of the invention before (A) and after (B) filtration.

DRWD FIGS. 18A and B are micrographs of gas-filled **liposomes** formed subsequent to filtering and autoclaving a lipid suspension, the micrographs having been taken before (A) and after (B) sizing by filtration of the gas-filled **liposomes**.

DETD At least in part, the gas impermeability of gas-filled **liposomes** has been found to be related to the gel state to liquid crystalline state phase transition temperature. By "gel state. . . is believed that, generally, the higher gel state to liquid crystalline state phase transition temperature, the more gas impermeable the **liposomes** are at a given temperature. See Table I below and Derek Marsh, CRC Handbook of Lipid Bilayers (CRC Press, Boca. . . also be noted that a lesser degree of energy can generally be used to release a therapeutic compound from gas-filled **liposomes** composed of lipids with a lower gel state to liquid crystalline state phase transition temperature.

DETD . . . various lipids will be readily apparent to those skilled in the art and are described, for example, in Gregoriadis, ed., **Liposome** Technology, Vol. I, 1-18 (CRC Press, 1984).

DETD . . . of ultrasound. The materials, such as lipids, used to construct the microspheres may be chosen for stability. For example, gas-filled **liposomes** composed of DSPC (distearoylphosphatidylcholine) are more stable than gas-filled **liposomes** composed of DPPC (dipalmitoylphosphatidylcholine) and that these in turn are more stable than gas-filled **liposomes** composed of egg **phosphatidylcholine** (EPC). Preferably, no more than about 50% of the microspheres rupture from the time of formation until the application of. . .

DETD . . . addition, it has been found that the incorporation of at least a small amount of negatively charged lipid into any **liposome** membrane, although not required, is beneficial to providing **liposomes** that do not have a propensity to rupture by fusing together. By at least a small amount, it is meant. . . phosphatidylserine and fatty acids. Most preferred for ability to rupture on application of resonant frequency ultrasound, echogenicity and stability are **liposomes** prepared from dipalmitoylphosphatidylcholine.

DETD **Liposomes** are a preferred embodiment of this invention since they are highly useful for entrapping gas. Additionally, gas-filled **liposomes** are preferred due to their biocompatibility and the ability to easily accommodate lipophilic therapeutic compounds that will easily cross cell membranes after the **liposomes** are ruptured. One skilled in the art, once armed with the present disclosure, would recognize that particular lipids may be. . .

DETD Where lipid material is used to create the microspheres, thus forming a **liposome**, a wide variety of lipids may be utilized in the construction

of the microspheres. The materials which may be utilized in preparing **liposomes** include any of the materials or combinations thereof known to those skilled in the art as suitable for **liposome** preparation. The lipids used may be of either natural or synthetic origin. The particular lipids are chosen to optimize the. . .

DETD The lipid in the gas-filled **liposomes** may be in the form of a single bilayer or a multilamellar bilayer, and are preferably multilamellar.

DETD Lipids which may be used to create **liposome** microspheres include but are not limited to: lipids such as fatty acids, lysolipids, **phosphatidylcholine** with both saturated and unsaturated lipids including dioleoylphosphatidylcholine; dimyristoylphosphatidylcholine; dipentadecanoylphosphatidylcholine, dilauroylphosphatidylcholine, dipalmitoylphosphatidylcholine; distearoylphosphatidylcholine; phosphatidylethanolamines such as dioleoylphosphatidylethanolamine; phosphatidylserine; phosphatidylglycerol; phosphatidylinositol,. . . acid; lipids bearing polymers such as polyethyleneglycol, chitin, hyaluronic acid or polyvinylpyrrolidone; lipids bearing sulfonated mono-, di-, oligo- or polysaccharides; **cholesterol**, **cholesterol** sulfate and **cholesterol** hemisuccinate; tocopherol hemisuccinate, lipids with ether and ester-linked fatty acids, polymerized lipids, diacetyl phosphate, stearylamine, cardiolipin, phospholipids with short chain. . .

DETD . . . 1,2-dioleoyloxy-3-(trimethylammonio)propane; and DOTB, 1,2-dioleoyl-3-(4'-trimethyl-ammonio)butanoyl-sn-glycerol may be used. In general the molar ratio of cationic lipid to non-cationic lipid in the **liposome** may be, for example, 1:1000, 1:100, preferably, between 2:1 to 1:10, more preferably in the range between 1:1 to 1:2.5. . .

DETD Solutions of lipids or gas-filled **liposomes** may be stabilized, for example, by the addition of a wide variety of viscosity modifiers, including, but not limited to. . . molecular weight ranges between 800 and 8000. Emulsifying and/or solubilizing agents may also be used in conjunction with lipids or **liposomes**. Such agents include, but are not limited to, acacia, **cholesterol**, diethanolamine, glyceryl monostearate, lanolin alcohols, lecithin, mono- and di-glycerides, mono-ethanolamine, oleic acid, oleyl alcohol, poloxamer, polyoxyethylene 50 stearate, polyoxyl 35. . . mono-palmitate, sorbitan monostearate, stearic acid, trolamine, and emulsifying wax. Suspending and/or viscosity-increasing agents that may be used with lipid or **liposome** solutions include, but are not limited to, acacia, agar, alginic acid, aluminum mono-stearate, bentonite, magma, carbomer 934P, carboxymethylcellulose, calcium and. . .

DETD . . . receptor may be provided to treat liver disease; thymidine kinase may be provided to treat ovarian cancer, brain tumors, or **HIV** infection; HLA-B7 may be provided to treat malignant melanoma; interleukin-2 may be provided to treat neuroblastoma, malignant melanoma, or kidney cancer; interleukin-4may be provided to treat cancer; **HIV** env may be provided to treat **HIV** infection; antisense ras/p53 may be provided to treat lung cancer; and Factor VIII may be provided to treat Hemophilia B.. . .

DETD . . . methotrexate (3-5'-dichloromethotrexate) with dialkyl esters, cytosine arabinoside with 5'-acylate, nitrogen mustard (2,2'-dichloro-N-methyldiethylamine), nitrogen mustard with aminomethyl tetracycline, nitrogen mustard with **cholesterol** or estradiol or dehydroepiandrosterone esters and nitrogen mustard with azobenzene.

DETD The size of drug containing **liposomes** can be adjusted, if desired, by a variety of procedures including extrusion, filtration, sonication, homogenization, employing a laminar stream of. . . Radiology, Vol. 22, pp. 47-55 (1987); PCT/US89/05040, U.S. Pat. No. 4,162,282; U.S. Pat. No. 4,310,505; U.S. Pat. No. 4,921,706; and **Liposome** Technology, Gregoriadis, G., ed., Vol. I, pp. 29-31, 51-67 and 79-108 (CRC Press Inc., Boca Raton, Fla. 1984). The disclosures. . .

DETD . . . In addition, dextrose may be preferably included in the media. Further solutions that may be used for administration of gas-filled **liposomes** include, but are not limited to, almond oil, corn oil, cottonseed oil, ethyl oleate, isopropyl myristate, isopropyl palmitate, mineral oil,. . .

DETD . . . potassium benzoate, potassium sorbate, sodium benzoate and sorbic acid. One or more antioxidants may further be included with the gas-filled **liposomes** to prevent oxidation of the lipid. Suitable antioxidants include tocopherol, ascorbic acid and ascorbyl palmitate.

DETD . . . in delivering therapeutics to a patient's lungs. Gas-filled microspheres of the present invention are lighter than, for example, conventional liquid-filled **liposomes** which generally deposit in the central proximal airway rather than reaching the periphery of the lungs. It is therefore believed. . .

DETD Specifically, in a preferred embodiment, a method for preparing a targeted drug delivery system comprising gas-filled **liposomes** of the subject invention comprises the steps of shaking an aqueous solution, comprising a lipid, in the presence of a gas at a temperature below the gel to liquid crystalline phase transition temperature of the lipid to form gas-filled **liposomes**, and adding a therapeutic compound. In another preferred embodiment, a method for preparing a targeted drug delivery system comprising gas-filled **liposomes** of the subject invention comprises the step of shaking an aqueous solution comprising a lipid and a therapeutic compound in. . . phase transition temperature of the lipid. In other embodiments, methods for preparing a targeted therapeutic drug delivery system comprising gas-filled **liposomes** comprise the steps of shaking an aqueous solution, comprising a lipid and a therapeutic compound, in the presence of a gas, and separating the resulting gas-filled **liposomes** for therapeutic use. **Liposomes** prepared by the foregoing methods are referred to herein as gas-filled **liposomes** prepared by a gel state shaking gas installation method and comprising a therapeutic compound, or as therapeutic containing gel state shaken gas instilled **liposomes**.

DETD The formation of gas-filled **liposomes** upon shaking can be detected by the presence of a foam on the top of the aqueous solution. This is. . .

DETD . . . in a 50 ml centrifuge tube may be vortexed for approximately 15-20 minutes or until the viscosity of the gas-filled **liposomes** becomes sufficiently thick so that it no longer clings to the side walls as it is swirled. At this time, the foam may cause the solution containing the gas-filled **liposomes** to raise to a level of 30 to 35 ml.

DETD . . . by one skilled in the art, once armed with the present disclosure. For example, in preferred embodiments, the concentration of 1,2-dipalimitoyl-**phosphatidylcholine** (DPPC) used to form gas-filled **liposomes** according to the methods of the present invention is about 20 mg/ml to about 30 mg/ml saline solution. The concentration. . .

DETD It will be understood by one skilled in the art, once armed with the present disclosure, that the lipids or **liposomes** may be manipulated prior and subsequent to being subjected to the methods of the present invention. For example, the lipid. . . then lyophilized, or hydrated, then processed through freeze and thaw cycles and then lyophilized, prior to the formation of gas-filled **liposomes**. In a most preferred embodiment, the lipid is hydrated and shaken, followed by at least one cycle of freezing in. . . liquid nitrogen and thawing, and then followed by lyophilization. Advantages to these treatments prior to the final formation of gas-filled **liposomes** include the transformation of the lipid to a solid form having a higher surface area, thus permitting better solubilization upon hydration and subsequently a higher yield of gas-filled **liposomes**.

DETD . . . various lipids will be readily apparent to those skilled in the art and are described, for example, in Gregoriadis, ed., **Liposome** Technology, Vol. I, 1-18 (CRC Press, 1984) and Derek Marsh, CRC Handbook of Lipid Bilayers (CRC Press, Boca Raton, Fla.. . .

DETD Conventional, aqueous-filled **liposomes** are routinely formed at a temperature above the gel to liquid crystalline phase transition temperature of the lipid, since they. . . the liquid crystalline state. See, for example, Szoka and Papahadjopoulos, Proc. Natl. Acad. Sci. 1978 75, 4194-4198. In contrast, the **liposomes** made according to preferred embodiments of the methods of the present invention are gas-filled, which imparts greater flexibility since gas is more compressible and compliant than an aqueous solution. Thus, the

gas filled **liposomes** may be utilized in biological systems which formed at a temperature below the phase transition temperature of the lipid, even. . .

DETD A preferred apparatus for producing the therapeutic containing gas-filled **liposomes** using a gel state shaking gas instillation process is shown in FIG. 9. A mixture of lipid and aqueous media is vigorously agitated in the process of gas installation to produce gas-filled **liposomes**, either by batch or by continuous feed. Referring to FIG. 9, dried lipids 51 from a lipid supply vessel 50. . .

DETD . . . a mixing vessel 66. Alternatively, the therapeutic compound may be added after the gas installation process, such as when the **liposomes** are coated on the outside with the therapeutic compound.

DETD . . . vessel 66 so that lipids are introduced in an aqueous solution. In the preferred embodiment of the method for making **liposomes**, the initial charge of solution 74 is such that the solution occupies only a portion of the capacity of the. . . vessel 66. Moreover, in a continuous process, the rates at which the aqueous lipid solution 74 is added and gas-filled **liposomes** produced are removed is controlled to ensure that the volume of lipid solution 74 does not exceed a predetermined percentage. . .

DETD . . . The shaking should be of sufficient intensity so that, after a period of time, a foam 73 comprised of gas-filled **liposomes** is formed on the top of the aqueous solution 74, as shown in FIG. 9. The detection of the formation. . .

DETD In a preferred embodiment of the apparatus for making gas-filled **liposomes** in which the lipid employed has a gel to liquid crystalline phase transition temperature below room temperature, a means for. . .

DETD After the shaking is completed, the gas-filled **liposome** containing foam 73 may be extracted from the mixing vessel 66. Extraction may be accomplished by inserting the needle 102. . . of the needle 102 is placed in the foam 73 may be used to control the size of the gas-filled **liposomes** extracted.

DETD . . . vessel 66 is pressurized, as previously discussed, the pressure of the gas 55 may be used to force the gas-filled **liposomes** 77 from the mixing vessel 66 to an extraction vessel 76 via conduit 70. In the event that the mixing. . . the foam 73 into the extraction vessel 76, as shown in FIG. 9. From the extraction vessel 76, the gas-filled **liposomes** 77 are introduced into vials 82 in which they may be shipped to the ultimate user. A source of pressurized gas 56 may be connected to the extraction vessel 76 as aid to ejecting the gas-filled **liposomes**. Since negative pressure may result in increasing the size of the gas-filled **liposomes**, positive pressure is preferred for removing the gas-filled **liposomes**.

DETD Filtration is preferably carried out in order to obtain gas-filled **liposomes** of a substantially uniform size. In certain preferred embodiments, the filtration assembly contains more than one filter, and preferably, the filters are not immediately adjacent to each other, as illustrated in FIG. 12. Before filtration, the gas-filled **liposomes** range in size from about 1 micron to greater than 60 microns (FIGS. 15A and 16A). After filtration through a single filter, the gas-filled **liposomes** are generally less than 10 microns but particles as large as 25 microns in size remain. After filtration through two filters (10 micron followed by 8 micron filter), almost all of the **liposomes** are less than 10 microns, and most are 5 to 7 microns (FIGS. 15B and 16B).

DETD . . . accomplished by incorporating a filter element 72 directly onto the end of the extraction tube 67 so that only gas-filled **liposomes** below a pre-determined size are extracted from the mixing vessel 66. Alternatively, or in addition to the extraction tube filter 72, gas-filled **liposome** sizing may be accomplished by means of a filter 80 incorporated into the conduit 79 that directs the gas-filled **liposomes** 77 from the extraction vessel 76 to the vials 82, as shown in FIG. 9. The filter 80 may contain. . .

DETD In addition to filtering, sizing may also be accomplished by taking advantage of the dependence of gas-filled **liposome** buoyancy on size. The gas-filled **liposomes** have appreciably lower density than water and hence will float to the top of the mixing vessel 66. Since the largest

liposomes have the lowest density, they will float most quickly to the top. The smallest **liposomes** will generally be last to rise to the top and the non gas-filled lipid portion will sink to the bottom. This phenomenon may be advantageously used to size the gas-filled **liposomes** by removing them from the mixing vessel 66 via a differential flotation process. Thus, the setting of the vertical location of the extraction tube 66 within the mixing vessel 66 may control the size of the gas-filled **liposomes** extracted; the higher the tube, the larger the gas-filled **liposomes** extracted. Moreover, by periodically or continuously adjusting the vertical location of the extraction tube 67 within the mixing vessel 66, the size of the gas-filled **liposomes** extracted may be controlled on an on-going basis. Such extraction may be facilitated by incorporating a device 68, which may. . .

DETD . . . based microspheres. In general, the greater the intensity of the shaking energy, the smaller the size of the resulting gas-filled **liposomes**.

DETD The current invention also includes novel methods for preparing drug-containing gas-filled **liposomes** to be dispensed to the ultimate user. Once gas-filled **liposomes** are formed, they can not be sterilized by heating at a temperature that would cause rupture. Therefore, it is desirable to form the gas-filled **liposomes** from sterile ingredients and to perform as little subsequent manipulation as possible to avoid the danger of contamination. According to. . . accomplished, for example, by sterilizing the mixing vessel containing the lipid and aqueous solution before shaking and dispensing the gas-filled **liposomes** 77 from the mixing vessel 66, via the extraction vessel 76, directly into the barrel 104 of a sterile syringe. . . shown in FIG. 10, without further processing or handling; that is, without subsequent sterilization. The syringe 100, charged with gas-filled **liposomes** 77 and suitably packaged, may then be dispensed to the ultimate user. Thereafter, no further manipulation of the product is required in order to administer the gas-filled **liposomes** to the patient, other than removing the syringe from its packaging and removing a protector (not shown) from the syringe. . . Moreover, the pressure generated when the syringe plunger 106 is pressed into the barrel 104 will cause the largest gas-filled **liposomes** to collapse, thereby achieving a degree of sizing without filtration.

DETD Where it is desired to filter the gas-filled **liposomes** at the point of use, for example because they are removed from the extraction vessel 76 without filtration or because. . . syringe 100 may be fitted with its own filter 108, as shown in FIG. 10. This results in the gas-filled **liposomes** being sized by causing them to be extruded through the filter 108 by the action of the plunger 106 when the gas-filled **liposomes** are injected. Thus, the gas-filled **liposomes** may be sized and injected into a patient in one step.

DETD . . . the lipid solution is extruded through a filter and the lipid solution is heat sterilized prior to shaking. Once gas-filled **liposomes** are formed, they may be filtered for sizing as described above. These steps prior to the formation of gas-filled **liposomes** provide the advantages, for example, of reducing the amount of unhydrated lipid and thus providing a significantly higher yield of gas-filled **liposomes**, as well as and providing sterile gas-filled **liposomes** ready for administration to a patient. For example, a mixing vessel such as a vial or syringe may be filled. . . sterilized within the mixing vessel, for example, by autoclaving. Gas may be instilled into the lipid suspension to form gas-filled **liposomes** by shaking the sterile vessel. Preferably, the sterile vessel is equipped with a filter positioned such that the gas-filled **liposomes** pass through the filter before contacting a patient.

DETD Where sterilization occurs by a process other than heat sterilization at a temperature which would cause rupture of the gas-filled **liposomes**, sterilization may occur subsequent to the formation of the gas-filled **liposomes**, and is preferred. For example, gamma radiation may be used before and/or after gas-filled **liposomes** are formed.

DETD FIG. 18 illustrates the ability of gas-filled **liposomes** to successfully form after autoclaving, which was carried out at

Further, after the extrusion and sterilization procedure, the shaking step yields gas-filled **liposomes** with little to no residual anhydrous lipid phase. FIG. 18A shows gas-filled **liposomes** generated after autoclaving but prior to filtration, thus resulting in a number of gas-filled **liposomes** having a size greater than 10 μm. FIG. 18B shows gas-filled **liposomes** after a filtration through a 10 μm "NUCLEPORE" filter, resulting in a uniform size around 10 μm.

DETD Certain embodiments of the present invention are directed to drug delivery systems comprising gas-filled **liposomes** prepared by vacuum drying gas instillation methods and having encapsulated therein a therapeutic (that is, drug containing), such **liposomes** sometimes being referred to herein as drug containing vacuum dried gas instilled **liposomes**. The present invention is further directed to drug delivery systems comprising drug containing gas-filled **liposomes** substantially devoid of liquid in the interior thereof.

DETD This method for preparing the **liposomes** of the subject invention comprises: (i) placing **liposomes** encapsulating a drug under negative pressure; (ii) incubating the **liposomes** under the negative pressure for a time sufficient to remove substantially all liquid from the **liposomes**; and (iii) instilling selected gas into the **liposomes** until ambient pressures are achieved. Methods employing the foregoing steps are referred to herein as the vacuum drying gas instillation methods for preparing drug containing **liposomes**.

DETD Apparatus is also provided for preparing the **liposomes** of the invention using the vacuum drying gas instillation methods, said apparatus comprising: (i) a vessel containing **liposomes** having encapsulated therein a drug; (ii) means for applying negative pressure to the vessel to draw liquid from the **liposomes** contained therein; (iii) a conduit connecting the negative pressurizing means to the vessel, the conduit directing the flow of said liquid; and (iv) means for introducing a gas into the **liposomes** in the vessel.

DETD The vacuum drying gas instillation method employed to prepare both the subject gas-filled **liposomes** prepared by the vacuum drying gas instillation method, and the gas-filled **liposomes** substantially devoid of liquid in the interior thereof, contemplates the following process. First, in accordance with the process, the drug containing **liposomes** are placed under negative pressure (that is, reduced pressure or vacuum conditions). Next, the **liposomes** are incubated under that negative pressure for a time sufficient to remove substantially all liquid from the **liposomes**, thereby resulting in substantially dried **liposomes**. By removal of substantially all liquid, and by substantially dried **liposomes**, as those phrases are used herein, it is meant that the **liposomes** are at least about 90% devoid of liquid, preferably at least about 95% devoid of liquid, most preferably about 100%. . . . devoid of liquid. Although the liquid is removed, the drug, with its higher molecular weight, remains behind, encapsulated in the **liposome**. Finally, the **liposomes** are instilled with selected gas by applying the gas to the **liposomes** until ambient pressures are achieved, thus resulting in the subject drug containing vacuum dried gas instilled **liposomes** of the present invention, and the drug containing gas-filled **liposomes** of the invention substantially devoid of liquid in the interior thereof. By substantially devoid of liquid in the interior thereof, as used herein, it is meant **liposomes** having an interior that is at least about 90% devoid of liquid, preferably at least about 95% devoid of liquid,. . .

DETD Unexpectedly, the drug containing **liposomes** prepared in accordance with the methods of the present invention possess a number of surprising yet highly beneficial characteristics. The **liposomes** of the invention exhibit intense echogenicity on ultrasound, will rupture on application of peak resonant frequency ultrasound (as well as. . . pressure, and/or generally possess a long storage life, either when stored dry or suspended in a liquid medium. The gas-filled **liposomes** also have the advantages, for example, of stable particle size, low toxicity and compliant membranes. It is believed that the flexible membranes of the gas-filled **liposomes** may be useful in aiding the accumulation or

unexpected is the ability of the **liposomes** during the vacuum drying gas instillation process to fill with gas and resume their original circular shape, rather than irreversibly. . .

DETD The echogenicity of the **liposomes** and the ability to rupture the **liposomes** at the peak resonant frequency using ultrasound permits the controlled delivery of drugs to a region of a patient by allowing the monitoring of the **liposomes** following administration to a patient to determine the presence of **liposomes** in a desired region, and the rupturing of the **liposomes** using ultrasound to release the drugs in the region. Preferably, the **liposomes** of the invention possess a reflectivity of greater than 2 dB, preferably between about 4 dB and about 20 dB. Within these ranges, the highest reflectivity for the **liposomes** of the invention is exhibited by the larger **liposomes**, by higher concentrations of **liposomes**, and/or when higher ultrasound frequencies are employed. See FIG. 13, which is a graphical representation of the dB reflectivity of gas-filled **liposomes** substantially devoid of liquid in the interior thereof prepared by the vacuum drying gas instillation method, without any drugs encapsulated therein. Preferably, the **liposomes** of the invention have a peak resonant frequency of between about 0.5 mHz and about 10 mHz. Of course, the peak resonant frequency of the gas-filled **liposomes** of the invention will vary depending on the diameter and, to some extent, the elasticity of the **liposomes**, with the larger and more elastic **liposomes** having a lower resonant frequency than the smaller and more elastic **liposomes**.

DETD The stability of the **liposomes** of the invention is also of great practical importance. The subject **liposomes** tend to have greater stability during storage than other gas-filled **liposomes** produced via known procedures such as ressurization or other techniques. At 72 hours after formation, for example, conventionally prepared gas ontaining **liposomes** often are essentially devoid of gas, the as having diffused out of the **liposomes** and/or the **liposomes** having ruptured and/or fused, resulting in a concomitant loss in reflectivity. In comparison, drug containing gas-filled **liposomes** of the present invention generally have a shelf life stability of greater than about three weeks, preferably a shelf life. . .

DETD Also unexpected is the ability of the **liposomes** during the vacuum drying gas instillation process to fill with gas and resume their original circular shape, rather than collapse. . .

DETD The drug containing **liposomes** subjected to the vacuum drying gas instillation method of the invention may be prepared using any one of a variety of conventional **liposome** preparatory techniques which will be apparent to those skilled in the art. Although any of a number of varying techniques can be employed, preferably the drug containing **liposomes** are prepared via microemulsification techniques. The **liposomes** produced by the various conventional procedures can then be employed in the vacuum drying gas instillation method of the present invention, to produce the drug containing **liposomes** of the present invention.

DETD The materials which may be utilized in preparing **liposomes** to be employed in the vacuum drying gas instillation method of the present invention include any of the materials or combinations thereof known to those skilled in the art as suitable for **liposome** construction.

DETD **Liposomes** may be prepared prior to gas installation using any one of a variety of conventional **liposome** preparatory techniques which will be apparent to those skilled in the art. These techniques include freeze-thaw, as well as techniques. . . solvent infusion, microemulsification, spontaneous formation, solvent vaporization, French pressure cell technique, controlled detergent dialysis, and others, each involving preparing the **liposomes** in various fashions in a solution containing the desired therapeutic so that the therapeutic is encapsulated in, enmeshed in, or attached the resultant **liposome**. Alternatively, therapeutics may be loaded into the **liposomes** using pH gradient techniques which, as those skilled in the art will recognize, is particularly applicable to therapeutics which either. . .

DETD To prepare the drug containing **liposomes** for vacuum drying gas installation, and by way of general guidance, dipalmitoylphosphatidylcholine **liposomes**, for example, may be prepared by suspending dipalmitoylphosphatidylcholine lipids in phosphate buffered saline or water containing the drug to be. . . required for transition of the dipalmitoylphosphatidylcholine lipids from a gel state to a liquid crystalline state, to form drug containing **liposomes**.

DETD To prepare multilamellar vesicles of a rather heterogeneous size distribution of around 2 microns, the **liposomes** may then be mixed gently by hand while keeping the **liposome** solution at a temperature of about 50° C. The temperature is then lowered to room temperature, and the **liposomes** remain intact. Extrusion of dipalmitoylphosphatidylcholine **liposomes** through polycarbonate filters of defined size may, if desired, be employed to make **liposomes** of a more homogeneous size distribution. A device useful for this technique is an extruder device (Extruder Device.TM., Lipex Biomembranes,. . .

DETD . . . which are sparingly soluble in aqueous media, such drugs may be mixed with the lipids themselves prior to forming the **liposomes**. For example, amphotericin may be suspended with the dried lipids (e.g., 8:2 molar ratio of egg **phosphatidylcholine** and **cholesterol** in chloroform and mixed with the lipids). The chloroform is then evaporated (note that other suitable organic solvents may also. . . This process may be used for a variety of lipophilic drugs such as corticosteroids to incorporate lipophilic drugs into the **liposome** membranes. The resulting **liposomes** are then dried, subjected to the vacuum gas instillation method as described above.

DETD Alternatively, and again by way of general guidance, conventional freeze-thaw procedures may be used to produce either oligolamellar or **unilamellar** dipalmitoylphosphatidylcholine **liposomes**. After the freeze-thaw procedures, extrusion procedures as described above may then be performed on the **liposomes**.

DETD The drug containing **liposomes** thus prepared may then be subjected to the vacuum drying gas instillation process of the present invention, to produce the drug containing vacuum dried gas instilled **liposomes**, and the drug containing gas-filled **liposomes** substantially devoid of liquid in the interior thereof, of the invention. In accordance with the process of the invention, the drug containing **liposomes** are placed into a vessel suitable for subjecting to the **liposomes** to negative pressure (that is, reduced pressure or vacuum conditions). Negative pressure is then applied for a time sufficient to remove substantially all liquid from the **liposomes**, thereby resulting in substantially dried **liposomes**. As those skilled in the art would recognize, once armed with the present disclosure, various negative pressures can be employed, the important parameter being that substantially all of the liquid has been removed from the **liposomes**. Generally, a negative pressure of at least about 700 mm Hg and preferably in the range of between about 700. . . pressure) applied for about 24 to about 72 hours, is sufficient to remove substantially all of the liquid from the **liposomes**. Other suitable pressures and time periods will be apparent to those skilled in the art, in view of the disclosures. . .

DETD Finally, a selected gas is applied to the **liposomes** to instill the **liposomes** with gas until ambient pressures are achieved, thereby resulting in the drug containing vacuum dried gas instilled **liposomes** of the invention, and in the drug containing gas-filled **liposomes** substantially devoid of liquid in the interior thereof. Preferably, gas instillation occurs slowly, that is, over a time period of. . .

DETD . . . be apparent to those skilled in the art, the gas chosen being only limited by the proposed application of the **liposomes**.

DETD The above described method for production of **liposomes** is referred to hereinafter as the vacuum drying gas instillation process.

DETD If desired, the **liposomes** may be cooled, prior to subjecting the **liposomes** to negative pressure, and such cooling is preferred. Preferably, the **liposomes** are cooled to below 0° C., more preferably to between about -10° C. and about -20° C., and most preferably to -10° C., prior to subjecting the **liposomes** to negative pressure. Upon reaching the desired negative pressure, the

liposomes temperature is then preferably increased to above 0° C., more preferably to between about 10° C. and about 20° C., and most preferably to 10° C., until substantially all of the liquid has been removed from the **liposomes** and the negative pressure is discontinued, at which time the temperature is then permitted to return to room temperature.

DETD If the **liposomes** are cooled to a temperature below 0° C., it is preferable that the vacuum drying gas instillation process be carried out with **liposomes** either initially prepared in the presence of cryoprotectants, or **liposomes** to which cryoprotectants have been added prior to carrying out the vacuum drying gas instillation process of the invention. Such cryoprotectants, while not mandatorily added, assist in maintaining the integrity of **liposome** membranes at low temperatures, and also add to the ultimate stability of the membranes. Preferred cryoprotectants are trehalose, glycerol, polyethyleneglycol. . .

DETD It has also been surprisingly discovered that the **liposomes** of the invention are highly stable to changes in pressure. Because of this characteristic, extrusion of the **liposomes** through filters of defined pore size following vacuum drying and gas instillation can be carried out, if desired, to create **liposomes** of relatively homogeneous and defined pore size.

DETD As another aspect of the invention, useful apparatus for preparing the drug containing vacuum dried gas instilled **liposomes**, and the drug containing gas-filled **liposomes** substantially devoid of liquid in the interior thereof, of the invention is also presented. Specifically, there is shown in FIG. 14 a preferred apparatus for vacuum drying **liposomes** and instilling a gas into the dried **liposomes**. The apparatus is comprised of a vessel 8 for containing drug containing **liposomes** 19. If desired, the apparatus may include an ice bath 5 containing dry ice 17 surrounding the vessel 8. The ice bath 5 and dry ice 17 allow the **liposomes** to be cooled to below 0° C. A vacuum pump 1 is connected to the vessel 8 via a conduit. . .

DETD In order to prevent liquid removed from the **liposomes** from entering the pump 1, a series of traps are connected to the conduit 15 to assist in collecting the liquid (and liquid vapor, all collectively referred to herein as liquid) drawn from the **liposomes**. In a preferred embodiment, two traps are utilized. The first trap is preferably comprised of a flask 7 disposed in. . . material. The ice baths 2 and 4 assist in collecting any liquid and condensing any liquid vapor drawn from the **liposomes** for collection in the traps. In preferred embodiments of the present invention the ice traps 2 and 4 are each. . .

DETD . . . upstream of the vessel 8 to allow a selected gas to be introduced into the vessel 8 and into the **liposomes** 19 from gas bottle 18.

DETD Apparatus of the present invention are utilized by placing the drug containing **liposomes** 19 into vessel 8. In a preferable embodiment, ice bath 5 with dry ice 17 is used to lower the temperature of the **liposomes** to below 0° C., more preferably to between about -10° C. and about -20° C., and most preferably to -10°. . . by ice bath 2 with dry ice 17, together or individually condense liquid vapor and trap liquid drawn from the **liposomes** so as to prevent such liquids and liquid vapor from entering the vacuum pump 1. In preferred embodiments of the. . . desired negative pressure is generally maintained for at least 24 hours as liquid and liquid vapor is removed from the **liposomes** 19 in vessel 8 and frozen in vessels 3 and 7. Pressure within the system is monitored using manometer 6. . . for about 24 to about 72 hours, at which time substantially all of the liquid has been removed from the **liposomes**. At this point, stopcock 10 is slowly closed and vacuum pump 1 is turned off. Stopcock 14 is then opened. . . into the system from gas bottle 18 through stopcock 14 via conduit 15 to instill gas into the drug containing **liposomes** 19 in vessel 8. Preferably the gas instillation occurs slowly over a time period of at least about 4 hours,. . .

DETD The drug containing vacuum dried gas instilled **liposomes** and the drug containing gas-filled **liposomes** substantially devoid of liquid in the interior thereof, of the present invention, have superior

characteristics as drug delivery vehicles.

DETD The gas-filled **liposomes** prepared according to the ethods of the present invention are believed to differ from the **liposomes** of the prior art in a number of respects, both in physical and in functional characteristics. For example, the **liposomes** of the invention are substantially devoid of liquid in the interior thereof. By definition, **liposomes** in the prior art have been characterized by the presence of an aqueous medium. See, e.g., Dorland's Illustrated Medical Dictionary, p. 946, 27th ed. (W. B. Saunders Company, Philadelphia 1988). Moreover, the present **liposomes** surprisingly exhibit intense echogenicity on ultrasound, are susceptible to rupture upon application of ultrasound at the peak resonant frequency of the **liposomes**, and possess a long storage life, characteristics of great benefit to the use of the **liposomes** as drug delivery systems.

DETD . . . for the controlled delivery of drugs to a region of a patient comprising: (i) administering to the patient the gas-filled **liposomes** prepared by vacuum drying gas instillation methods and having encapsulated therein a drug, and/or gas-filled **liposomes** substantially devoid of liquid in the interior thereof and having encapsulated therein a drug; (ii) monitoring the **liposomes** using ultrasound to determine the presence of the **liposomes** in the region; and (iii) rupturing the **liposomes** using ultrasound to release the drugs in the region.

DETD There are various other applications for **liposomes** of the invention, beyond those described in detail herein. Such additional uses, for example, include such applications as hyperthermia potentiators. . .

DETD . . . therapeutic. Examples 11-20 are prophetic examples that describe the preparation, testing and use of the drug-containing vacuum dried, gas instilled **liposomes**, the gas-filled **liposomes** being substantially devoid of any liquid in the interior thereof. Examples 21-28 are actual examples that illustrate the preparation and testing of the gas-filled **liposomes** prepared by shaking an aqueous solution comprising a lipid in the presence of a gas. Examples 29-36 are actual examples that illustrate the preparation and sizing of gas-filled **liposomes** prepared by filtering and autoclaving a lipid suspension, followed by shaking the lipid solution. The following examples should not be. . .

DETD . . . in gas-filled microspheres and that ultrasound can be used to release a therapeutic from a gas-filled microsphere. As shown below, **liposomes** entrapping water and DNA failed to release the genetic material after exposure to the same amount of ultrasonic energy. The. . .

DETD Gas-filled **liposomes** were synthesized as follows: Pure dipalmitoylphosphatidylcholine (DPPC), Avanti Polar Lipids, Alabaster, Ala., was suspended in normal saline and then Extruded. . . micron polycarbonate filters (Nuclepore, Costar, Pleasanton, Calif.) using an Extruder Device (Lipex Biomembranes, Vancouver, Canada) at 800 p.s.i. The resulting **liposomes** were then dried under reduced pressure as described in U.S. Ser. No. 716,899, filed Jun. 18, 1991, which is hereby incorporated by reference in its entirety. After thorough drying the dried **liposomes** were then slowly filled with nitrogen gas, as described in U.S. Ser. No. 716,899. After equilibration with ambient pressure, the resulting **liposomes** were suspended in saline solution (0.9% NaCl) and shaken vigorously.

DETD The resulting gas-filled **liposomes** were then tested for size by Coulter Counter (Bedfordshire, England). The machine was calibrated using the calibration procedure described in the reference manual supplied with the Coulter Counter. The gas-filled **liposome** solution was diluted with Isoton II and placed in a glass container and was stirred at the 3 position of. . .

DETD . . . then placed on the glass slide and viewed under different magnifications. This technique results in sizing not only of gas-filled **liposomes**, but also lipid particles.

DETD The gas-filled **liposomes** were scanned by sonic energy using both an Acoustic Imaging Model 5200 clinical ultrasound device (Acoustic Imaging Technologies Corp., Phoenix,. . .

DETD . . . energies of pulsed sound used in these experiments over the

gas-filled **liposomes** remains constant despite continual scanning for 60 minutes at a power setting between 4.5-8.4 mW and an acoustic intensity of. . .

DETD Solutions of gas-filled **liposomes** were also subjected to continuous wave ultrasound energy (Table IV) applied with a Rich-Mar Therapeutic ultrasound apparatus model RM-25 (Rich-Mar. . . produced using continuous wave ultrasound. It was found that continuous wave energy of sound caused the gas inside the gas-filled **liposomes** to escape from the **liposomes**, thus rupturing the **liposomes**. It took approximately 20-30 minutes to completely destroy the gas-filled **liposomes** in a solution of saline at 5 watts of power and at 1 MHz. It took approximately 5 minutes to destroy the gas-filled **liposomes** at 10 watts and at 1 MHz. When the gas-filled **liposomes** were examined by light microscopy before and after application of high energy ultrasound the spherical shape of the gas-filled **liposomes** disappeared after exposure.

DETD Gas-filled microspheres were then tested for their ability to deliver DNA in a series of experiments. **Liposomes** were prepared from DPPC as described above except that 2 μg of DNA on a 7000 bp plasmid (pCH110: Pharmacia LKB Biotechnology, Piscataway, N.J.), in 1 cc of normal saline were added during resuspension of the dried DPPC. Gas-filled **liposomes** were then prepared as described above. After resuspension of the gas-filled **liposomes**, external unentrapped DNA was removed by affinity chromatography. The suspension of gas-filled **liposomes** and DNA was eluted through a column (DNA specific Sephadex®) using a peristaltic pump (Econopump, Bio-Rad Laboratories, Hercules, Calif.). The. . .

DETD **Liposomes** filled with water were also prepared as described above to entrap DNA except that the drying gas instillation step was omitted. Unentrapped DNA was removed via chromatography. The gas-filled **liposomes** were then scanned ultrasonically as described above. The gas-filled **liposomes** containing DNA were similarly echogenic to pulsed ultrasound as described above. After scanning with continuous wave ultrasound as described above,. . .

DETD After treatment with continuous wave ultrasound, a propidium iodide dimer assay for free DNA (i.e., DNA external to the gas-filled **liposomes**) was performed and compared to control gas-filled **liposomes** containing DNA (i.e., not exposed to continuous wave ultrasound).

DETD Gas-filled **liposomes** containing DNA not exposed to high energy ultrasound retained a substantial amount of their DNA internally as indicated by the. . .

DETD . . . then extruded through a 2 μm filter using an Extruder Device (Lipex Biomembranes, Inc., Vancouver, BC). Then positively charged gas-filled **liposomes** are prepared according to the procedure provided in U.S. Ser. No. 717,084, filed Jun. 18, 1991, which is hereby incorporated. . .

DETD The resulting dried, positively charged gas-filled **liposomes** are rehydrated by adding PBS, saline or other appropriate buffered solution (such as HEPES buffer); a vortexer may be used. . . is added and the mixture is again shaken. Since the DNA is attached to the surface of the cationic gas-filled **liposomes**, unattached DNA may be removed with filtering or selective chromatography. Essentially all of the DNA binds up until the point. . .

DETD The resulting DNA coated **liposomes** are then dried and gas instilled to create DNA-containing gas-filled **liposomes**. The resulting **liposomes** are then exposed to continuous wave ultrasound and tested for rupturing by reflectivity and absorbance on ultrasound.

DETD . . . that cationic lipids binding DNA provide an advantage, for example, since once sonic energy has disrupted the membrane of the **liposome**, the hydrophobic groups help the DNA to integrate into cells aiding passage through cell membranes and subcellular compartments.

DETD . . . have an advantage of neutralizing the negative charge of DNA and amphipathicity. When these cationic lipids are released from the **liposomes**, since the lipids are amphiphilic and the cell membrane is soluble, they tend to facilitate passage of the DNA into. . .

DETD **liposomes** composed of a 1:1 molar ratio of DOTMA and DPPC are prepared and coated with DNA encoding an HLA (major histocompatibility complex) gene, HLA-B7. The DNA-coated **liposomes** are injected intravenously into a patient with metastatic melanoma involving the soft tissues. Continuous wave 1.0 megahertz ultrasound energy is. . .

DETD Anti-sense oligonucleotides to the Ras oncogene are entrapped within **liposomes** composed of polyethyleneglycol-dipalmitoylphosphatidylethano lamine. These **liposomes** are injected i.v. in a patient with metastatic colon cancer. Continuous wave 1.0 megahertz ultrasound energy is applied to the. . .

DETD Gas-filled microspheres are made as described above using egg **phosphatidylcholine** and DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoium chloride, to bind YAC expression vectors carrying the dystrophin gene. The microspheres are injected i.v. into a. . .

DETD Cationic gas-filled **liposomes** with three types of surface-bound antisense DNAs are synthesized as described above. The antisense DNAs are targeted against genes encoding c-myc, c-myb, smooth muscle growth factor, and endothelial cell growth factor. The gas-filled **liposomes** are administered intra-arterially to an angioplasty site. 5 megahertz of continuous wave ultrasound is then applied to the angioplasty site.. . .

DETD . . . suspension is swirled for two additional hours, while allowing the suspension to cool to room temperature, to form drug containing **liposomes**.

DETD The **liposomes** thus prepared are placed in a vessel in an apparatus similar to that shown in FIG. 14, cooled to about -10° C., and are then subjected to high negative vacuum pressure. The temperature of the **liposomes** is then raised to about 10° C. High negative vacuum pressure is maintained for about 48 hours. After about 48. . . 4 hours after which time the pressure is returned to ambient pressure. The resulting drug containing vacuum dried gas instilled **liposomes**, the gas-filled **liposomes** being substantially devoid of any liquid in the interior thereof, are then suspended in 10 cc of phosphate buffered saline. . .

DETD To test the **liposomes** of Example 11 ultrasonographically, a 250 mg sample of these **liposomes** is suspended in 300 cc of non-degassed phosphate buffered saline. The **liposomes** are then scanned in vitro at varying time intervals with a 7.5 mHz transducer using an Acoustic Imaging Model 5200. . . Phoenix, Ariz.) and employing the system test software to measure dB reflectivity. The system is standardized prior to testing the **liposomes** with a phantom of known acoustic impedance. Good dB reflectivity of the **liposomes** is shown.

DETD . . . the suspension is swirled for about two additional hours, while allowing the suspension to cool to room temperature, to form **liposomes**.

DETD The **liposomes** thus prepared are then vacuum dried and gas instilled, substantially following the procedures shown in Example 11, resulting in drug containing vacuum dried gas instilled **liposomes**, the gas-filled **liposomes** being substantially devoid of any liquid in the interior thereof. The **liposomes** are then suspended in 10 cc of phosphate buffered saline and vortexed, and then stored at about 4° C. for several weeks. Prior to use, the gas-filled **liposomes** are extruded through a 10 μm polycarbonate filter (Nuclepore, Costar, Pleasanton, Calif.) by injection through a syringe with a filter. . .

DETD To test the **liposomes** of Example 13 ultrasonographically, the procedures of Example 12 are substantially followed. Good dB reflectivity of the **liposomes** is shown.

DETD . . . the suspension is swirled for about two additional hours, while allowing the suspension to cool to room temperature, to form **liposomes**.

DETD The **liposomes** thus prepared are then vacuum dried and gas instilled, substantially following the procedures shown in Example 11, resulting in drug containing vacuum dried gas instilled **liposomes**, the gas-filled **liposomes** being substantially devoid of any liquid in the interior thereof. The **liposomes** are then suspended in 10 cc of phosphate buffered saline, and then stored at about 4° C. for several weeks.

DETD To test **liposomes** of Example 15 ultrasonographically, the procedures

of Example 11 are substantially followed. Good dispersivity of the **liposomes** is shown.

DETD In order to test the stability of the drug containing **liposomes** of the invention, the **liposomes** suspension of Example 11 are passed by hand through a 10 micron polycarbonate filter in a syringe as shown in FIG. 10. Af ter extrusion treatment, the **liposomes** are studied ultrasonographically, as described in Example 12. Surprisingly, even after extrusion, the **liposomes** of the invention subst antially retain their echogenicity.

DETD The **liposomes** of Example 11 are scanned by ultrasound using transducer frequencies varying from 3 to 7.5 mHz. The results indicate that. . .

DETD A patient with cancer is given an intravenous drug containing vacuum dried gas instilled **liposomes**, the gas-filled **liposomes** being substantially devoid of any liquid in the interior thereof. The drug contained in the **liposomes** is adriamycin. As the intravenous injection is administered, the tumor is scanned ultrasonographically and via an automated software program, and the resonant frequency of the **liposomes** is determined. Ultrasonic energy is then focused into the tumor at the peak resonant frequency of the **liposomes**. The amount of ultrasonic energy is insufficient to cause any appreciable tissue heating (that is, no change in temperature greater than 2° C.), however, this energy is sufficient to cause the **liposomes** to pop and release the adriamycin at the tumor site. In so doing, local drug delivery is accomplished using the **liposomes** with ultrasound.

DETD In a patient with a severe localized fungal infection, drug containing vacuum dried gas instilled **liposomes**, the gas-filled **liposomes** being substantially devoid of any liquid in the interior thereof, are injected intravenously and ultrasound is used in a fashion substantially similar to that described in Example 19 to accomplish local drug delivery. The drug amphotericin-B, which the **liposomes** contain, is effectively delivered to the site of the infection.

DETD In order to prepare gas-filled **liposomes**, fifty mg of 1,2-Dipalmitoyl-Sn-Glycero-3-Phosphocholine (MW: 734.05, powder, Lot No. 160pc-183) (Avanti-Polar Lipids, Alabaster, Ala.) was weighed and hydrated with 5.0. . .

DETD The gas-filled **liposomes** made via this new method were then sized by optical microscopy. It was determined that the largest size of the **liposomes** ranged from about 50 to about 60 μm and the smallest size detected was about 8 μm. The average size. . .

DETD The gas-filled **liposomes** were then filtered through a 10 or 12 μm "NUCLEPORE" membrane using a Swin-Lok Filter Holder, ("NUCLEPORE" Filtration Products, Costar. . . Cambridge, Mass.). The 10.0 μm filter was placed in the Swin-Lok Filter Holder and the cap tightened down securely. The **liposome** solution was shaken up and it was transferred to the 20 cc syringe via an 18 gauge needle. Approximately 12 ml of **liposome** solution was placed into the syringe, and the syringe was screwed onto the Swin-Lok Filter Holder. The syringe and the filter holder assembly were inverted so that the larger of the gas-filled **liposomes** vesicles could rise to the top. Then the syringe was gently pushed up and the gas-filled **liposomes** were filtered in this manner.

DETD The survival rate (the amount of the gas-filled **liposomes** that were retained after the extrusion process) of the gas-filled **liposomes** after the extrusion through the 10.0 μm filter was about 83-92%. Before hand extrusion, the volume of foam was about. . .

DETD The optical microscope was used again to determine the size distribution of the extruded gas-filled **liposomes**. It was determined that the largest size of the **liposomes** ranged from about 25 to about 30 μm and the smallest size detected was about 5 μgm. The average size. . .

DETD It was found that after filtering, greater than 90% of the gas-filled **liposomes** were smaller than 15 μm.

DETD . . . this solution was pipetted onto a slide and the solution was viewed under a microscope. The size of the gas-filled **liposomes** was then determined. It was determined that the largest size of the

liposomes was about 60 μm and the smallest size detected was about 20 μm. The average size ranged from about 30. . .

DETD . . . It was noted that very little foaming of the lipid (phase transition temp. =41° C.) did not appreciably form gas-filled **liposomes**. Optical microscopy revealed large lipidic particles in the solution. The number of gas-filled **liposomes** that formed at this temperature was less than 3% of the number that form at a temperature below the phase. . . C.). The solution was then vortexed for a duration of 10 minutes. After 10 minutes, it was noted that gas-filled **liposomes** formed.

DETD . . . then repeated eight times. The hydrated suspension was then vortexed for 10 minutes at an instrument setting of 6.5. Gas-filled **liposomes** were then detected as described in Example 21.

DETD It was determined that the largest size of the gas-filled **liposomes** with 1 mol% of sodium lauryl sulfate was about 75 μm and the smallest size detected was about 6 μm.. . . average size ranged from about 15 to about 40 μm. It was determined that the largest size of the gas-filled **liposomes** with 10 mol% of sodium lauryl sulfate was about 90 μm and the smallest size detected was about 6 μm.. . .

DETD The volume of foam in the solution containing gas-filled **liposomes** with 1 mol% sodium lauryl sulfate was about 15 ml and the volume of aqueous solution was about 3-4 ml. The volume of foam in the solution containing gas-filled **liposomes** with 10 mol% sodium lauryl sulfate was also about 15 ml and the volume of aqueous solution was about 3-4. . .

DETD This example determined whether sonication could be used to create gas-filled **liposomes**. 50 mg of lipid, 1,2-Dipalmitoyl-Sn-Glycero-3-Phosphocholine (Avanti-Polar Lipids, Alabaster, Ala.), was weighed out and hydrated with 5 ml of 0.9% NaCl.. . . sonicate for 10 minutes. Following sonication, the solution was viewed under an optical microscope. There was no evidence of gas-filled **liposomes** having been produced.

DETD . . . with this tip. After 10 minutes, the solution was viewed under the microscope. Again, there was no evidence of gas-filled **liposomes**.

DETD This example determined whether a lower concentration limit of the lipid would halt the production of gas-filled **liposomes**. Ten mg of 1,2-Dipalmitoyl-Sn-Glycero-3-Phosphocholine (Avanti-Polar Lipids, Alabaster, Ala.) was added to 10 ml of saline solution (0.9% w:v NaCl). The. . . minutes. The solution was viewed under an optical microscope for sizing. It was determined that the largest size of the **liposomes** ranged from about 30 to about 45 μm and the smallest size detected was about 7 μm. The average size. . .

DETD It appeared that the gas-filled **liposomes** were more fragile as they appeared to burst more rapidly than previously shown. Thus, it appears that concentration of the lipid is a factor in the generation and stability of gas-filled **liposomes**.

DETD Unfiltered gas-filled **liposomes** were drawn into a 50 ml syringe and passed through a cascade of a "NUCLEPORE" 10 μm filter and 8. . . be filtered through a stack of 10 μm and 8 μm filters that are immediately adjacent to each other. Gas-filled **liposomes** were passed through the filters at such a pressure whereby the flow rate was 2.0 ml min^{-1}. The subsequently filtered gas-filled **liposomes** were then measured for yield of gas-filled lipid microspheres which resulted in a volume of 80-90% of the unfiltered volume.

DETD The resulting gas-filled **liposomes** were sized by four different methods to determine their size and distribution. Sizing was performed on a Particle Sizing Systems. . . (Coulter Electronics Limited, Luton, Beds., England). As can be seen in FIGS. 15 and 16, the size of the gas-filled **liposomes** were more uniformly distributed around 8-10 μm as compared to the unfiltered gas-filled **liposomes**. Thus, it can be seen that the filtered gas-filled **liposomes** are of much more uniform size.

DETD . . . 60 Hz.) (Scientific Industries, Inc., Bohemia, N.Y.) for 10 minutes or until a time that the total volume of gas-filled **liposomes** was at least double or triple the volume of the original aqueous lipid

devoid of anhydrous particulate lipid, and a large volume of foam containing gas-filled **liposomes** resulted. Thus, prior autoclaving does not affect the ability of the lipid suspension to form gas-filled **liposomes**. Autoclaving does not change the size of the **liposomes**, and it does not decrease the ability of the lipid suspensions to form gas-filled **liposomes**.

DETD . . . shaken at 300 r.p.m. for 30 minutes. The resultant agitation on the shaker table resulted in the production of gas-filled **liposomes**.

DETD . . . a gyrating otion for 15 minutes. After vigorous mixing, the centrifuge tube was removed, and it was noted that gas-filled **liposomes** had formed.

DETD . . . to room temperature (approximately 20° C.), the tube was shaken forcefully by hand for ten minutes. Upon ceasing agitation, gas-filled **liposomes** were formed.

DETD Gas-filled **liposomes** were produced from DPPC as described in Example 32. The resultant unfiltered **liposomes** were drawn into a 50 ml syringe and passed through a cascade filter system consisting of a "NUCLEPORE" (Costar, Pleasanton,. . . a stacked 10 μm and 8 μm filtration assembly was used, with the two filters adjacent to one another. Gas-filled **liposomes** were passed through the filters at a pressure such that they were filtered a rate of 2.0 ml/min. The filtered gas-filled **liposomes** yielded a volume of 80-90% of the unfiltered volume.

DETD The resultant gas-filled **liposomes** were sized by four different methods to determine their size distribution. Sizing was performed on a Particle Sizing Systems (Santa. . . and a Coulter Counter (Coulter Electronics Limited, Luton, Beds., England). As illustrated in FIG. 18, the size of the gas-filled **liposomes** was more uniformly distributed around 8-10 μm as compared to the unfiltered gas-filled **liposomes**.

CLM What is claimed is:

. . . therapeutic delivery system of claim 1 wherein the lipid is selected from the group consisting of distearoylphosphatidylcholine, dipalmitoylphosphatidylcholine and egg **phosphatidylcholine**.

16. A therapeutic delivery system of claim 1 wherein the microspheres comprise gas-filled **liposomes** substantially devoid of liquid in the interior thereof and having encapsulated therein a therapeutic compound.

17. A therapeutic delivery system of claim 1 wherein the microspheres comprise gas-filled **liposomes** prepared by a vacuum drying gas instillation method and having encapsulated therein a therapeutic compound.

18. A therapeutic delivery system of claim 1 wherein the microspheres comprise gas-filled **liposomes** prepared by a gel state shaking gas instillation method.

19. A drug delivery system comprising a gas-filled **liposome** prepared by a vacuum drying gas instillation method and having encapsulated therein a drug, wherein said gas-filled **liposome** has an interior volume of at least about 50% gas, and wherein said drug is selected from the group consisting. . .

20. A drug delivery system of claim 19 wherein said **liposomes** are comprised of lipid materials selected from the group consisting of fatty acids, lysolipids, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, **phosphatidylcholine**, phosphatidic acid, sphingomyelin, **cholesterol**, **cholesterol** sulfate, **cholesterol** hemisuccinate, tocopherol hemisuccinate, phosphatidylethanolamine, phosphatidylinositol, glycosphingolipids, glucolipids, glycolipids, sulphatides, lipids bearing sulfonated mono-, di-, oligo- or polysaccharides, lipids with ether. . .

21. A drug delivery system of claim 20 wherein said **liposomes** are comprised of dipalmitoylphosphatidylcholine.

22. A drug delivery system of claim 19 wherein said **liposomes** are

filled with a gas selected from the group consisting of air, nitrogen, carbon dioxide, oxygen, argon, xenon, helium, and. . .
23. A drug delivery system of claim 22 wherein said **liposomes** are filled with nitrogen gas.

24. A drug delivery system of claim 19 wherein said **liposomes** are stored suspended in an aqueous medium.

25. A drug delivery system of claim 19 wherein said **liposomes** are stored dry.

26. A drug delivery system of claim 19 wherein said **liposomes** have a stability of at least about three weeks.

27. A drug delivery system of claim 19 wherein said **liposomes** have a reflectivity of at least about 2 dB.

28. A drug delivery system of claim 27 wherein said **liposomes** have a reflectivity of between about 2 dB and about 20 dB.

29. A drug delivery system comprising a gas-filled **liposome** wherein said gas-filled **liposome** has an interior volume of at least about 50% gas and comprises a drug encapsulated therein, wherein said drug is. . .
30. A drug delivery system of claim 29 wherein said **liposomes** are comprised of lipid materials selected from the group consisting of fatty acids, lysolipids, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, **phosphatidylcholine**, phosphatidic acid, sphingomyelin, **cholesterol**, **cholesterol** hemisuccinate, tocopherol hemisuccinate, phosphatidylethanolamine, phosphatidylinositol, glycosphingolipids, glucolipids, glycolipids, sulphatides, lipids bearing sulfonated mono-, di-, oligo- or polysaccharides, lipids with ether. . .
31. A drug delivery system of claim 30 wherein said **liposomes** are comprised of dipalmitoylphosphatidylcholine.

32. A drug delivery system of claim 29 wherein said **liposomes** are filled with a gas selected from the group consisting of air, nitrogen, carbon dioxide, oxygen, argon, xenon, helium, and. . .
33. A drug delivery system of claim 32 wherein said **liposomes** are filled with nitrogen gas.

34. A drug delivery system of claim 29 wherein said **liposomes** are stored suspended in an aqueous medium.

35. A drug delivery system of claim 29 wherein said **liposomes** are stored dry.

36. A drug delivery system of claim 29 wherein said **liposomes** have a shelf life stability of at least about three weeks.

37. A drug delivery system of claim 29 wherein said **liposomes** have a reflectivity of at least about 2 dB.

38. A drug delivery system of claim 37 wherein said **liposomes** have a reflectivity of between about 2 dB and about 20 dB.

39. A drug delivery system prepared by a method comprising the steps of: (i) placing under negative pressure **liposomes** having encapsulated therein a drug, wherein said drug is selected from the group consisting of antineoplastic agents; blood products; biological. . . neuromuscular blockers; sedatives; local anesthetics; general anesthetics; radioactive compounds; monoclonal antibody; genetic material; prodrugs; and combinations thereof; (ii) incubating said **liposomes** under the negative pressure for a time sufficient to remove substantially all liquid from said **liposomes**; and (iii) instilling gas

[illegible] said **liposomes** under ambient pressures are removed to produce a drug delivery system comprising gas-filled **liposomes** having an interior volume of at least about 50% gas.

. . . microsphere comprises at least one lipid selected from the group consisting of dipalmitoylphosphotdylcholine, dipalmitoylphosphatidylethanolamine, and a phosphalidic acid, and said **liposome** further comprising polyethylene glycol.

46. The drug delivery system of claim 19 comprising dipalmitoylphosphatidylethanolamine, said **liposome** further comprising polyethylene glycol.

47. A therapeutic delivery system of claim 1 wherein said gas-filled microsphere is a lipid-containing **unilamellar** microsphere.

48. A drug delivery system of claim 19 wherein said gas-filled **liposome** is **unilamellar**.

49. A drug delivery system of claim 29 wherein said gas-filled **liposome** is **unilamellar**.

50. A drug delivery system of claim 39 wherein said gas-filled **liposomes** are **unilamellar**.

51. A therapeutic delivery system of claim 47 wherein said **unilamellar** microsphere comprises a phospholipid.

52. A drug delivery system of claim 48 wherein said **unilamellar liposome** comprises a phospholipid.

53. A drug delivery system of claim 49 wherein said **unilamellar liposome** comprises a phospholipid.

54. A drug delivery system of claim 50 wherein said **unilamellar liposomes** comprise a phospholipid.

62. A drug delivery system of claim 48 wherein said **liposome** comprises polymerized lipids.

63. A drug delivery system of claim 49 wherein said **liposome** comprises polymerized lipids.

64. A drug delivery system of claim 50 wherein said **liposomes** comprise polymerized lipids.

66. A drug delivery system of claim 48 wherein said **liposome** further comprises polyethylene glycol.

67. A drug delivery system of claim 49 wherein said **liposome** further comprises polyethylene glycol.

68. A drug delivery system of claim 50 wherein said **liposomes** further comprise polyethylene glycol.

75. A drug delivery system of claim 39 wherein prior to said placing said **liposomes** under the negative pressure, said **liposomes** are allowed to cool to a temperature between about -10° C. and about -20° C., wherein the negative pressure is. . . mm Hg, wherein said incubating step is for about 24 to about 72 hours, wherein during said incubating step said **liposomes** are allowed to warm to a temperature between about 10° C. and about 20° C., wherein said instilling step occurs over a period of about 4 to about 8 hours, and wherein during said instilling step said **liposomes** are allowed to warm to ambient temperature.

L17 ANSWER 12 OF 15 USPATFULL on STN
97:65876 Biologic bioadhesive compositions containing fibrin glue and **liposomes**, methods of preparation and use.
Marx, Gerard, New York, NY, United States
New York Blood Center, Inc., New York, NY, United States (U.S. corporation)
US 5651982 19970729
APPLICATION: US 1995-465888 19950606 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention describes a biologically compatible bioadhesive sealant composition comprising fibrin glue and **liposomes** for use in mammals, including humans. Fibrin glue of the invention comprises fibrinogen and thrombin which are mixed together in various modes with **liposomes** and applied to a site of injury, to a wound, or to a surgical or nonsurgical incision or opening. In accordance with the invention, the **liposomes** are embedded within the fibrin glue after coagulation has occurred, and may release bioactive substances contained within their aqueous interiors to promote healing and protection during the recovery process. The bioadhesive composition of the invention promises to maintain hemostasis after surgeries and improves upon existing glues or gel formulations due to its complete biological compatibility, its formation in situ, and its provision of bioactive therapeutics via entrapped **liposomes** directly to the site. Long-lasting biophysical and biomechanical properties as well as therapeutic value are imparted to the fibrin glue components by the **liposome** component of the composition. The biocompatible fibrin glue and **liposome** composition is also amenable for fabrication into films, coatings, or membranes for in vitro and in vivo uses.

TI Biologic bioadhesive compositions containing fibrin glue and **liposomes**, methods of preparation and use

AB The present invention describes a biologically compatible bioadhesive sealant composition comprising fibrin glue and **liposomes** for use in mammals, including humans. Fibrin glue of the invention comprises fibrinogen and thrombin which are mixed together in various modes with **liposomes** and applied to a site of injury, to a wound, or to a surgical or nonsurgical incision or opening. In accordance with the invention, the **liposomes** are embedded within the fibrin glue after coagulation has occurred, and may release bioactive substances contained within their aqueous interiors. . . gel formulations due to its complete biological compatibility, its formation in situ, and its provision of bioactive therapeutics via entrapped **liposomes** directly to the site. Long-lasting biophysical and biomechanical properties as well as therapeutic value are imparted to the fibrin glue components by the **liposome** component of the composition. The biocompatible fibrin glue and **liposome** composition is also amenable for fabrication into films, coatings, or membranes for in vitro and in vivo uses.

SUMM The invention describes bioadhesive sealant compositions containing fibrin glue comprising human-source components in combination or admixture with **liposomes**, and methods for making the compositions. Compositions and methods of the invention are suitable for accelerating and ameliorating the healing. . .

SUMM . . . most commonly bovine, is routinely used in commercially-available fibrin glues. Human thrombin can be employed in the formulation of the **liposome**-containing fibrin glue bioadhesive, as can other appropriate catalyzing enzymes, such as reptilase or select venoms (Fenton II, J. W. et. . .

SUMM The fibrinogen preparations used in the fibrin glue and **liposome** compositions can be virally inactivated by one or more methods prior to their employment in the invention (e.g. Examples 1-3).

SUMM . . . fibrinogen are also envisioned. For example, fibrinogen made by recombinant techniques could also be employed in the fibrin glue and **liposome** composition. Molecular techniques available for the production of recombinant fibrinogen include the use of COS-1 or Hep G2 cells transfected. . . types of cells. Normal or mutant recombinant fibrinogens may be employed in fibrin glue compositions formulated with

SUMM the types of **liposomes** as described herein.

SUMM . . . time because of the general risks and problems of infection from pooled blood products contaminated with lipid-enveloped viruses such as **HIV**, associated with AIDS, and the hepatitis-causing viruses such as HBV and HCV (also known as non A-non B hepatitis virus),. . . human use in the United States, is obtained from bovine sources which do not appear to carry significant risks for **HIV** and hepatitis, although other bovine pathogens may be present.

SUMM **Liposomes**

SUMM **Liposomes** are **unilamellar** or multilamellar lipid vesicles which entrap a significant fraction of aqueous solution. The vesicular microreservoirs of **liposomes** can contain a variety of water-soluble materials, which are thus suspended within the emulsion (reviewed in G. Gregorius (Ed.), 1991, **Liposome** Technology, Vols. I, II, III, CRC Press, Boca Raton, Fla.; M. J. Ostro (Ed.), 1983, **Liposome** Preparations: Methods & Mechanisms, Marcel Dekker Inc. New York; Davis S. S. and Walker I. M., 1987, Methods in Enzymology,. . . 52:139-149; Patel H. M., 1985, Trans. Biochem. Soc., 13:513-516; Ostro M. J., 1987 (Jan.), Sci. Am., 91). The preparation of **liposomes** and the variety of uses of **liposomes** in biological systems have been disclosed in U.S. Pat. No. 4,708,861 to M. C. Popescu et al., U.S. Pat. No.. . . U.S. Pat. No. 4,235,871 to D. P. Papahadjopoulos and F. C. Szoka, Jr., P. R. Cullis et al., 1987, In: **Liposomes** as Pharmaceuticals, M. J. Ostro, Ed., Marcel Dekker, New York, 39-72, and H. G. Weder et al., 1986, In: **Liposomes** as drag carriers, K. H. Schmidt, Ed., Thieme, Stuttgart, 26-39.

SUMM **Liposomes** are formed by mixing long chain carboxylic acids, amines, and **cholesterol**, as well as phospholipids, in aqueous buffers. The organic components spontaneously form multilamellar bilayer structures (i.e. **liposomes**). Depending on their composition and storage conditions, **liposomes** exhibit varying stabilities. **Liposomes** serve as models of cell membranes and also have been used as drug delivery systems (M. Schafer-Korting et al., 1989, J. Am. Acad. Dermatol., 21:1271-1275). Most attempts to use **liposomes** as drug delivery vehicles have envisioned **liposomes** as entities which circulate in blood, to be taken up by certain cells or tissues in which their degradation would. . . release their internal aqueous drug-containing contents. In an effort to aid in their up-take by a given target tissue, some **liposomes** have been "tailored" by binding specific antibodies or antigens to the outer surface. **Liposomes** have also been devised as controlled release systems for delivery of their contents in vivo (H. M. Patel, 1985, Biochem. Soc. Transactions, 13:513-516). Compositions in which **liposomes** containing biologically active agents are maintained and immobilized in polymer matrices, such as methycellulose, collagen, and agarose, for sustained release of the **liposome** contents, are described in U.S. Pat. No. 4,708,861 to M. C. Popescu et al.

SUMM The present invention affords a new generation of virally inactivated bioadhesive sealant compositions comprising fibrin glue and **liposomes** whose advantages and uses will become apparent from the following objectives of the invention and disclosure. The present invention establishes a safe and unique fibrin glue and **liposome** formulation for widespread use and numerous surgical and nonsurgical applications.

SUMM It is an object of the present invention to provide a biological composition in which fibrin glue and **liposomes** are combined together to form a distinctive and novel bioadhesive for administration to animals, including humans. The final fibrin glue and **liposome** bioadhesive formulation may be achieved in a number of different ways, such as by premixing at least one of the fibrin glue components with **liposomes** prior to application or administration, and then adding the remaining component(s) to form in situ the final **liposome**-containing fibrin glue.

SUMM It is another object of the invention to provide **liposomes** tailored for use in conjunction with fibrin glue in various modes. In one mode, a fibrinogen solution can be mixed with **liposomes** and stored in the cold (i.e., at 4° C.), or frozen (i.e., at -30° C.), or lyophilized. When desired, the fibrinogen/**liposome** mixture is thawed

of a surgical or non-surgical opening or wound, thereby forming **liposome**-containing fibrin glue. Alternatively, **liposomes** can be pre-mixed with a solution of thrombin and stored cold, frozen, or lyophilized. When desired, thawed, warmed, or reconstituted fibrinogen is mixed with a thrombin and **liposome** mixture at the site of administration, thereby forming **liposome**-containing fibrin glue.

SUMM It is a further object of the invention to provide a stable fibrin glue and **liposome** sealing matrix which is inexpensive and safe and can be easily applied over a surgical or nonsurgical wound or opening,. . . mammal, including humans, to promote, accelerate, and protect sealing and healing at and around the site. The fibrin glue and **liposome** formulation remains at the site of application long enough to promote and protect the healing process. The fibrin glue is. . .

SUMM It is another object of the invention to provide a fibrin glue-containing **liposome** composition in which medicaments or bioactive additives are encapsulated or entrapped in the **liposomes**. The **liposomes** serve as vehicles or carriers of the medicaments and additives at an injury site, surgical or nonsurgical opening, or wound. The contents of the **liposomes** are released at the site after application, either in a spontaneous or controlled fashion.

SUMM Yet another object of the invention is to provide fibrin glue and **liposome** bioadhesive compositions in which the **liposomes** add desirable properties to the glue components. The storage characteristics of both thrombin and fibrinogen may be modulated and significantly improved by **liposome** components. Biophysical properties of the fibrin glue composition, such as rate of gelation, viscoelasticity, and tensile strength, may be modulated and further improved by the **liposome** components of the composition.

SUMM . . . surgery, or a wound while simultaneously providing the slow or rapid release of bioactive ingredients which are contained in the **liposomes** of the fibrin glue/**liposome** formulation. The formulation may comprise a number of different types of **liposomes**, each containing a different bioactive agent. Alternatively, the formulation may comprise a number of **liposomes** of a particular type, each containing different additives.

SUMM Another object of the invention is to provide a biologically compatible sealing agent comprising fibrin glue combined with **liposomes** to favor and maintain hemostasis following its use, even in heparinized individuals and in individuals suffering from coagulopathies. In addition, the fibrin glue and **liposome**-containing bioadhesive system of the invention promises to reduce the incidence of fistula formation and to decrease postoperative infections, tissue necrosis,. . .

SUMM . . . another objective of the invention is to provide fibrin glue compositions containing virally inactivated fibrinogen and thrombin preparations admixed with **liposomes** and other glue components from human and animal sources to yield a virally inactivated fibrin glue and **liposome** preparation. Such virally inactivated fibrin glue and **liposome** compositions afford safer and contaminant-free preparations which can be admixed and employed in accordance with the invention.

DRWD FIG. 1. Size (i.e., diameter in microns) distribution of neutral **liposomes** (Type A), determined in a Coulter particle analyzer. The size distribution of the prepared **liposomes** was not significantly altered when the **liposomes** were formed in a buffer in the presence of 2 mM zinc chloride.

DRWD . . . (made from cryoprecipitate ("Cryo") as the source of fibrinogen and thrombin with and without 5% (v/v) of neutral (Type A) **liposomes** ("Lipo A"), amino (Type B) **liposomes** ("Lipo B"), or carboxylic acid (Type C) **liposomes** ("Lipo C"). Fibrin glue was formulated using 10 U/mL of thrombin and 10 mM Ca^{2+}.

DRWD . . . (i.e., Fraction I paste of Cohn preparation) and thrombin without ("Cohn 1") and with 5% (v/v) of neutral (Type A) **liposomes**, "Lipo A"; amino (Type B) **liposomes**, "Lipo B"; or carboxylic acid (Type C) **liposomes**, "Lipo C". Fibrin glue was formulated using 10 U/mL of thrombin and 10 mM Ca^{2+}.

DRWD . . . of fibrin glue formulated with cryoprecipitate ("Cryo") and

liposomes ("Cryo+Lipo A", filed circles), amino (Type B) **liposomes** ("Cryo+Lipo B", fined triangles), and carboxylic acid (Type C) **liposomes** ("Cryo+Lipo C", filled squares).

DRWD . . . concentration) and thrombin without (open circles) and with 50 µL (filled circles) or 100 µL (filled squares) of Type A **liposomes** ("Lipo A") added to the fibrin glue mixture (300 µL total).

DRWD . . . as a fibrinogen source, and with fibrin glue formulated from cryoprecipitate as a fibrinogen source in combination with Type A **liposomes** ("Cryo & Lipo A").

DRWD FIG. 8. Breaking strength (BS) of fibrin glue film without ("Neat") and with Type A, B, or C **liposomes** (8% by volume), ("Lipo A, Lipo B, Lipo C", respectively). Dimensions of fibrin glue film: 2 mm thick, 1 cm. . .

DRWD . . . 9. Percent elongation ("% Elong") prior to breaking of fibrin glue film without and with Type A, B, or C **liposomes** (8% by volume), ("Lipo A, Lipo B, Lipo C", respectively). Initial dimensions: 2 mm thick, 1 cm wide. Fibrin glue. . .

DRWD FIG. 10. Photograph of fibrin glue (45 mg/mL fibrinogen) containing Type A **liposomes** (8% by volume) formulated into a film. The strip of fibrin glue and **liposome** film is being twisted 90°.

DRWD FIG. 11. Photograph of fibrin glue (45 mg/mL fibrinogen) containing Type A **liposomes** (8% by volume) flexibly coating aluminum foil as a solid substrate.

DRWD FIG. 12. Breaking strength (BS) of fibrin glue ("FG") mixed with bone fragments ("Bone") without or with Type A **liposomes**, 8% by volume, ("Lipo A"). Bone fragments not longer than 2 mM were mixed with fibrin glue without or with **liposomes** and the matrix was allowed to set for 1 hour at 37° C. in a moist environment. Fibrin glue components:. . .

DETD The fibrinogen for use in producing the fibrin glue-containing **liposome** composition of the invention may be prepared by employing starting materials of varying purities and by following a number of. . .

DETD . . . describes the range of amounts, and the preferred and more preferred amounts of fibrinogen and thrombin (or other activating enzymes), **liposomes**, and calcium used to formulate fibrin glue in which **liposomes** are embedded. The resulting concentration of fibrinogen in the final fibrin glue composition is about 10-90 mg/mL, preferably about 30-60. . .

DETD TABLE 1

	Fibrinogen	Thrombin	**Liposomes**	Calcium (Ca(II))
	mg/mL	U/mL	µL/mL	mM
Suitable Range	10-90	1-200	20-300	1-30
Preferred Range	30-60	5-100	50-100	10-15
Optimal Range	40	10	75	10

DETD The fibrin glue and **liposome** bioadhesive composition of the invention constitutes a new generation of biological and bioactive sealants, adhesives, or material for fabricating films. . . (e.g. viruses and the like) found in the final composition. In addition, the production of the present fibrin glue containing **liposomes** is economical to make and use.

DETD **Liposomes** for use in fibrin glues in general, and in the mixture with fibrinogen in particular, may be formed by a variety of techniques. It is important that the **liposomes**, however they are produced, do not adversely modify the physical properties of the fibrin glue once they are embedded in the glue. As will be clear from the present invention,

deter from the clot forming and bioadhesive attributes of the fibrin glue. In fact, the **liposomes** themselves may favorably modulate the biophysical properties of the fibrin glue by increasing or decreasing the rate of clot formation. . . 1988, Thrombos. Haemostas., 59:500-503; Marx G. and Blankenfeld, A., 1993, Blood Coag. and Fibrinolysis, 4:73-78). It is envisioned that the **liposomes** may be formulated to entrap a component or components which could modulate the final fibrin glue characteristics. For example, some or all of the **liposomes** for use in the fibrin glue composition of the invention may be prepared to contain aprotinin or other anti-proteases, such as ε-amino caproic acid (EACA) in their aqueous phase compartment, which, when released at the site of fibrin glue-**liposome** composition during glue degradation, would slow the rate of protein degradation, thus prolonging the lifetime and viability of the fibrin. . .

DETD The fibrin glue formulated in the presence of **liposomes** maintains its mechanical properties as determined by breaking strength or tensile strength studies and viscoelasticity studies (e.g., FIGS. 2-6). In the present invention the quality of the biophysical parameters is not significantly modified by the **liposomes** in the bioactive composition. Indeed, the **liposomes** are even suitable for improving the quality of the fibrin glue formulated to include such **liposomes**.

DETD The basic constituents of **liposomes** are various saturated or unsaturated lipids or phospholipids, with or without the addition of **cholesterol** and other constituents, such as aliphatic compounds having either amino or carboxylic acid groups. **Liposomes** of several types suitable for use in the present invention may be prepared by a variety of techniques that may ultimately influence **liposome** morphology, type, and size. Many techniques for preparing **liposomes** have been described (Szoka Jr. and Papahaddjopoulos, D. (1980), Ann. Rev. Biophys. Bioengineering, 9:467-508; Gregorius G. (Ed). (1983) **Liposome** Technology, Vols. I, II, III., CRC Press, Boca Raton, Fla. (1991); Ostro (Ed)., **Liposome** Preparations: Methods & Mechanisms, Marcel Dekker Inc. N.Y.; Davis, S. S. and Walker, I. M., (1987), Methods in Enzymology 149: 51-64). These methods are applicable for producing **liposomes** for embedding into the fibrin glue to be applied to a specific tissue site. As but one example, a technique for producing **liposomes** for use in the invention is by ethanol injection. In this technique, equimolar quantities of **cholesterol** and hydrogenated lecithin are mixed and warmed to 60° C. in ethanol to form a solution, and the solution is. . . emulsion is incubated 60° C. for one hour, centrifuged at 2000×g for 5 minutes, and the supernatant is removed. The **liposomes** are suspended in Tris-saline buffer and stored at 4° C.

DETD **Liposomes** of several types are suitable for use in the present invention. For example, neutral **liposomes** designated as Type A (described in Example 5) are formulated with equimolar amounts of **cholesterol** and hydrogenated lecithin and are referred to as neutral **liposomes**. As described in Example 6, Type B **liposomes** are formulated with 50% **cholesterol**, 40% hydrogenated lecithin, and 10% stearyl amine on a molar basis. As a consequence of their composition, Type B **liposomes** have free amine groups on their surfaces; such groups on Type B **liposomes** are potentially capable of affecting certain biophysical parameters of the fibrin glue composition. Type C **liposomes**, described in Example 7, are formulated with 50% **cholesterol**, 40% hydrogenated lecithin, and 10% stearic acid on a molar basis. Type D **liposomes** are formulated with 50% **cholesterol**, 40% hydrogenated lecithin, and 10% diethylstearylamine on a molar basis. Depending on pH, exemplary **liposomes** of the B and C types contain electrically-charged chemical moieties on their surfaces.

DETD Large **liposomes** (e.g. multilamellar vesicles having a diameter size range of 0.1 to 5 to >10 μm, and large **unilamellar** vesicles having a diameter size of ≥0.06 μm) and small **liposomes** (e.g. small **unilamellar** vesicles having a diameter size of about 0.02 to 0.05 μm) may be employed in the present invention and may. . . produced by conventional methods as previously indicated. Although those skilled in the art will appreciate that virtually all types of **liposomes** are

suitable for use in the present invention, some **liposome** types may have particular properties which make them especially conducive to forming a stable and effective fibrin glue-**liposome** bioadhesive. For example, as described above, neutral **liposomes** of 5 μm diameter with a good load capacity (e.g. at least about 10% to 20% aqueous phase) may be. . .

DETD In accordance with the invention, the contents of the **liposomes** are routinely entrapped in the aqueous phase, rather than within the membrane bilayer of the **liposome**. The amount of aqueous phase containing bioactive material incorporated into the aqueous interior compartments of the **liposomes** for effective use in the invention was tested by forming Type A **liposomes** in Tris buffer (Tris-saline buffer, pH 7.4) containing 2 mM $Zn^{2}+$. The $Zn^{2}+$ which remained after washing the **liposomes** was measured by X-ray fluorescence (Gorodetsky, R., Mou, X., Blankenfeld, A., and Marx, G., 1993, Amer. J. Hematol., 42:278-283). From these measurements, it was estimated that the **liposomes** contained, on a volume per volume basis, about 10% to 20% aqueous phase, which is indicative of the "load" of the **liposomes**. The loaded **liposomes** were stable at both 4° C. and 22° C., and they retained their aqueous phases quite well over a period of several weeks. Such $Zn^{2}+$ containing **liposomes** were used in in vivo studies in accordance with the invention (Example 10).

DETD Knowing the **liposome** load allows the calculation of the effective concentration or fraction of reagent(s) entrapped (i.e., contained) within the aqueous phase for. . . the orginal amount of the aqueous starting material used and the final amount of material that is contained in the **liposome**. As a specific but non-limiting example, the $Zn^{2}+$ solution served as a quantifiable marker for the entrapped contents of the **liposomes**. Thus, the amount of $Zn^{2}+$ in the starting solution is determined (e.g., 130 ppm). The **liposomes** are formulated to entrap a portion of the $Zn^{2}+$ solution. The fractional **liposome** volume is determined from a particle counter. The **liposomes** so formulated are washed in buffer. After washing, the aqueous phase is removed and the amount of $Zn^{2}+$ that has been entrapped into the **liposome** is measured.

DETD Using 300 μL of **liposomes** containing 2 mM $Zn^{2}+$ solution in their interiors would translate overall into 60 μM $Zn^{2}+$ in 1 mL of fibrin glue at and around the site of the wound. The **liposomes** can fuse with cells at the site of the wound and thereby merge their aqueous contents with the contents of cells. Thus, the aqueous phase of the **liposome** is delivered to the wound site where it subsequently diffuses into and around the site of application.

DETD Another aspect of the invention involves the use of fibrin glue containing light sensitive or photoactivatable **liposomes** (LSL), which may provide more controlled release of their contents into the environment over time. Such **liposomes** can also be prepared to contain bioactive materials, additives, and medicaments, and are kept in a light-protected (e.g. filtered) container. . . LSL, thereby causing them to release their contents into the surroundings at the site of application. Light sensitive and photoactivatable **liposomes** are prepared essentially as described for non-light sensitive **liposomes**; they are virtually identical, but they provide for manipulated, light-controlled release of their contents. Light sensitive **liposomes** may be prepared by using lecithins of retinoic acid (such as 1,2-diretinoyl-sn-3-glycerophosphocholine (DRPC), 2-retinoylisolecithin (LRPC) or 1-palmitoyl-2-retinoyl-sn-3-glycerophosphocholine (PRPC) in the lipid and **cholesterol** mixture, as described (Pidgeon, C. and Hunt, C. A., 1987, Methods in Enzymol. 149:99-111). Mixtures of DRPC/LRPC in ratio ranges of about 70:30 to about 30:70 are used to formulate light sensitive **liposomes**. Some formulations might include up to 40% added α-tocopherol (α-T) to help in forming the light-sensitive **liposomes**. Preparations of LSL would necessarily be kept in the dark to prevent light induced degradation of the **liposome** structures and the release of their aqueous compartments. The light-sensitive **liposomes** are mixed with fibrin glue components (i.e., fibrinogen or

thrombin, of the fibrin glue and **liposome** composition to allow their compartments to be released when desired by exposure to the chosen light source.

DETD Alternatively, the glue component, thrombin, is incorporated into the LSL. Such thrombin-containing **liposomes** are then mixed directly with fibrinogen in a light filtered container. The release of thrombin would be instigated by exposing. . .

DETD Bioactive agents contained in **liposomes** of the fibrin glue-containing **liposome** composition

DETD The **liposomes** of the invention are designed to contain, carry, and release biologically active agents in accordance with the internal load or capacity of the **liposomes**. It is envisioned that **liposomes** containing biologically active substances and medicaments and embedded in the fibrin glue will carry and release their contents at a. . . and protection process following all types of surgical or wound healing procedures and applications. Examples of applications for the "loaded" **liposomes** in fibrin glue include, but are not limited to, partial or complete replacement of sutures in skin grafts, bums or. . .

DETD A wide variety of biologically active agents as well as medicines and pharmaceuticals may be contained within the **liposomes** of the fibrin glue-**liposome** formulation. Examples of various agents to be entrapped in the **liposomes** include, but are not limited to, drags, neuroleptics, vitamins (e.g. Vitamin C, (i.e. ascorbic acid or ascorbate), Vitamin A, Vitamin. . . of some or all of the above may be used, when practical, as additives in the aqueous phase of the **liposomes** of the invention. In addition, lipophilic drugs or other compounds may be incorporated into the phospholipid membrane of the **liposomes**.

DETD The invention is suitable for multiple bioactive agents to be contained in **liposomes** used in the fibrin glue bioadhesive composition. Should such a utility be desired, two or more bioactive agents may be entrapped in one **liposome** type which forms an integral part of the fibrin glue, and becomes subsequently embedded or deposited in the glue clot. Alternatively, two or more different types of **liposomes** or mixtures of **liposome** populations, each of which entraps the same or different bioactive agents, may be embedded in the fibrin glue-**liposome** composition. Different preparations of **liposomes** may comprise monophasic lipid vesicles (i.e. those having **unilamellar** lipid bilayers) or plurilamellar vesicles (i.e. those having multilamellar lipid bilayers), such as have been described previously (M. Schafer-Korting et. . . U.S. Pat. No. 4,708,861 to M. C. Popsecu et al.). As envisioned for use in the present fibrin glue and **liposome** composition, one type of lipsomes (e.g. neutral **liposomes**) is formulated to entrap a particular bioactive material and a second type of **liposomes** (either the same type as the first or a different type) is formulated to entrap another bioactive material. Both types of **liposomes** containing their respective bioactive contents are mixed with the components comprising fibrin glue, and the resulting fibrin glue and **liposome** composition contains two types of **liposomes** capable of delivering their respective bioactive contents at the incision or wound or opening site. It is apparent that mixtures of different types of **liposomes** containing a variety of bioactive materials may be formulated and embedded in the composition.

DETD Fibrin glue-containing **liposome** formulations

DETD As described hereinbelow, **liposomes** may be suspended in either a fibrinogen or thrombin solution and stored at temperatures from about 4° C. to about 37° C. prior to use. Alternatively, individual mixtures of **liposomes** and either fibrinogen or thrombin preparations may be frozen at -70° C. or lyophilized by drying in vacuo at about. . . mixtures are reconstituted in water or buffer such as Tris-saline. All of the methods of storage result in viable, long-lasting **liposome** glue compositions following reconstitution of the stored materials.

DETD In accordance with the invention, **liposomes** formulated with fibrin glue in a variety of modes result in **liposome**-containing fibrin glue in which **liposomes** are embedded and deposited in the clotted fibrin glue bioadhesive. Because fibrin glue forms the environment for

deposition of the **liposomes**, the glue localizes the **liposomes** at the site or sites of application.

DETD . . . invention is that it is physiologically compatible with biological systems for in vivo use, such that both it and the **liposomes** contained therein, provide beneficial effects for the recipient animal without being toxic. Similarly, another advantage of the fibrin glue-**liposome** compositions is that the **liposome**-glue will remain in clotted form in the environment in which it is administered or applied due to the formulations of. . . and will withstand physiological body temperatures and the conditions of the host environment in vivo. Because they comprise phospholipids and **cholesterol**, the **liposomes** of the composition will also be naturally metabolized over time by absorption by cells and tissue (reviewed by Schater-Korting M.,. . . It is clear that the fibrin glue allows for controlled localization and the release of the contents of the embedded **liposomes** into the desired tissue site.

DETD In one embodiment of the invention, fibrinogen, thrombin, and **liposomes** are each stored separately, and then are mixed together when desired, for use to form the fibrin glue-**liposome** composition at the site of the surgical or nonsurgical wound or opening. By way of example, lyophilized fibrinogen (about 50-70. . . of 10 mM Tris-saline, pH 7.4) to form a fibrinogen solution and a thrombin solution. Thereafter, about 200 μL of **liposomes** were added to the fibrinogen solution which was mixed gently to form a fibrinogen-**liposome** suspension. The above solutions were formulated in syringes and the steps to mix the component solutions were carried out in syringes. The fibrinogen and **liposome** suspension were applied simultaneously, along with the thrombin solution, to the site of the wound. The fibrin glue that formed within minutes contained about 10% embedded **liposomes** and provided a **liposome**-containing bioadhesive at the wound site. On average, about 1% to about 20% (by volume), more preferably about 2% to about 15% (by volume), and most preferably, about 5% to about 10% (by volume) of **liposomes** were embedded in the fibrin glue to produce the fibrin glue and **liposome** bioadhesive of the invention. The amount of **liposomes** in the composition represent the volume per volume percentage of **liposomes** in the final fibrin glue and **liposome** formulation.

DETD In another embodiment, **liposomes** are pre-mixed with a solution of fibrinogen, and stored at 4° C. The **liposome** and fibrinogen mixture can be lyophilized, if desired, prior to storage. Prior to or at the time of use, the mixture of **liposomes** and fibrinogen is warmed to 37° C. and mixed with thrombin solution at the site of injury or opening, thereby forming the fibrin glue composition in which the **liposomes** are entrapped.

DETD In another embodiment, **liposomes** are pre-mixed with a solution of thrombin, and stored at 4° C. Prior to or at the time of use, the mixture of **liposomes** and thrombin is mixed with reconstituted fibrinogen solution at the site of the surgical or nonsurgical wound or opening, thereby forming **liposome**-containing fibrin glue.

DETD . . . thrombin solutions are each stored in separate receptacles or containers (e.g. syringes) prior to use or mixing in the fibrin glue-**liposome** composition. **Liposomes** may be suspended in a fibrinogen solution in a first syringe, and then mixed with the thrombin solution from a second syringe on or around the site of the wound or opening. Alternatively, **liposomes** may be suspended in a thrombin solution in a first syringe, and then mixed with fibrinogen solution from a second. . .

DETD In vivo and other uses of fibrin glue-containing **liposome** composition

DETD The fibrin glue-**liposome** composition of the present invention may be used for immediate or sustained release of a biologically active substance or medicament both in vitro and in vivo. For in vivo use at a surgical or nonsurgical site, the fibrin glue-**liposome** composition may be formulated in the number of ways elucidated above. In brief, the fibrin glue components and **liposomes**, however they are pre-mixed, may be added together at or over the wound site at the desired time of use. Consequently, the fibrin glue-**liposome** bioadhesive is formed in situ following the admixture and administration of all of the components at

biochemical action of fibrin glue mimics a part of a normal biological process, the fibrin glue-**liposome** composition may be used to promote hemostasis by controlling hemorrhaging, to seal and bond tissue, and to support wound healing. Similarly, the fibrin glue-containing **liposome** composition may be topically administered at the site of bums in which the release of antimicrobials, cell growth factors, and/or. . .

DETD Fibrin glue containing **liposomes** can be also be used to bind bone fragments. The bone-binding ability of the fibrin glue and **liposome** composition is very useful in bone reconstruction, as in plastic surgery or the repair of major bone breaks. For example, a bone fracture can be sealed with the fibrin glue and **liposome** composition so that the glue both seals the break and entraps and localizes the **liposomes** which are formulated to contain bone-specific growth factors. Upon slow dissolution of the fibrin glue at the site of the bone fracture, the **liposomes** release their entrapped growth factors and thus improve the rate and quality of the healing bone.

DETD . . . autologous bone from a patient can be ground or made into powder or the like, added to fibrinogen mixed with **liposomes**, and mixed into a paste. Thrombin is then mixed with the fibrinogen and **liposome** paste in an amount sufficient (i.e., 1 U/mL) to allow the paste to be applied to the desired locale, where the fibrin glue and **liposome** composition finally congeals. The amount of time for the congealing of the composition to occur can be controlled by adjusting the level of thrombin used. The **liposomes** can be formulated to contain bone growth factors (for example, as described in Sampath T. K. et al., 1992, J.. . . Sci. USA, 87:220) or antibiotics in their aqueous phases. One skilled in the art will appreciate that the types of **liposomes** (e.g., neutral or charged) and the choice of aqueous phase components can be chosen as desired.

DETD Fibrin glue containing **liposomes** can also be fabricated as a film or membrane. Such films or membranes are advantageous to cover large surface areas. In addition, the fibrin glue and **liposome** compositions can be employed to fabricate implantable devices which include not only films, but also foams or chunks of the congealed fibrin glue and **liposome** composition. The films and devices may be formed ex vivo by application as liquids or sprays for subsequent implantation or use in vivo after gelation. Such fibrin glue-containing **liposome** compositions may be also be used to coat devices, such as prosthethic devices, catheters, or valves, and the like, which. . .

DETD . . . and stretched up to 4 times their initial dimensions before breaking. Fibrin glue films containing Type A, B, or C **liposomes** which entrap biologically active compounds within their aqueous phases, are suitable for use in the invention. For example, fibrin glue film containing Type A, B, or C **liposomes** can be sprayed onto a hydrophobic surface, such as Parafilm (American Can Co.) to form a fibrin glue and **liposome** film or membrane, which does not adhere permanently to the surface. After setting or cross-linking for about 1 hour, the fibrin glue film formulated with its entrapped **liposomes** is peeled away from the parafilm surface and exhibits physical characteristics virtually identical to fibrin glue film formulated without **liposomes**. The combination of fibrin glue film and **liposomes** can impart beneficial biological effects to such films used as described above for formation in situ. The results of producing a film comprising the fibrin glue and **liposome** compositions is described in Example 12.

DETD Fibrin glue film containing **liposomes** can also be used to coat or to layer over a variety of materials used to make prosthetic devices for implantation. In an embodiment of this invention, fibrin glue containing **liposomes** can be sprayed or applied as liquid onto a metal surface or other substrate onto which the composition adheres tightly. For example, a fibrin glue film containing Type A **liposomes** sprayed onto aluminum foil bound very tightly. Alternately, the film could be formed by layering the fibrin glue and **liposome** mixture onto the surface or substrate. When aluminum foil was used as the substrate, the film (about 1 mm thick). . . serve to illustrate, but not to limit, the further embodiments of the invention in which Types A, B, or C **liposomes** are

prior to use in animals or humans.

DETD . . . of fibrinogen preparation are provided. Using cryoprecipitate as a source of fibrinogen is suitable for formulating the fibrin glue and **liposome** composition of the invention. Alternatively and oftentimes more preferably, fibrinogen is desired in a more purified or concentrated form, and. . .

DETD . . . Table 2). The cryoprecipitate was warmed to 37° C. for 15 minutes prior to use in the fibrin glue and **liposome** composition.

DETD . . . in the art will appreciate that cryoprecipitate prepared from fresh-frozen plasma is suitable for use in the bioadhesive composition containing **liposomes**, as is a more purified preparation of fibrinogen such as that obtained from, but not limited to, the Fraction I. . .

DETD . . . stored at -30° C. or was lyophilized. Thawed fibrinogen or fibrinogen reconstituted by adding water or buffer was mixed with **liposomes** of Types A, B, C, or D, as desired, without precipitation or gelling of the resulting fibrinogen and **liposome** mixture.

DETD Laboratory scale preparations of fibrinogen, including SD fibrinogen, for use in the bioadhesive **liposome** and fibrin glue compositions typically contained the following constituents as indicated. The fibrinogen constituents are provided as a guide and. . .

DETD . . . quenchers. The purified thrombin maintained its enzymatic activity, even following viral inactivation treatment. In addition, mixing purified thrombin alone with **liposomes** of Types A-C as disclosed herein did not significantly alter its enzymatic or clot-inducing activity.

DETD Preparation of **Liposomes**

DETD To prepare **liposomes** containing a different lipids and **cholesterol** via the ethanol injection technique, equimolar quantities of **cholesterol** (Chol) and hydrogenated lecithin (HL) were dissolved in 100 μL of absolute ethanol 100 μL chloroform and mixed at 60°. . . 10-fold larger concentration of Tris-saline buffer, pH 7.4, containing the material to be entrapped in the aqueous phase of the **liposome**. Some **liposomes** were also made with stearyl amine (B) or stearic acid (C) or diethylstearylamine added to the alcohol phase in one-tenth molar quantities relative to the amounts of Chol and HL used. Following the addition of all of the **liposome** reagents to the aqueous phase, the mixture was incubated an additional 1 hour at 60° C., and then treated in an ultrasonic bath for 5 minutes. After ultrasonification, **liposomes** were cooled to 22° C. for 1 hour, centrifuged at 2000×g for 10 minutes, washed in Tris-saline buffer, pH 7.4, and re-centrifuged two times more before storage at 4° C. Evaluation of the prepared **liposomes** in a Coulter particle sizer (the Coulter Company) showed unimodal distribution of particles with a diameter range of from about. . .

DETD Preparation of "Neutral" or "Type A" **Liposomes**

DETD For the preparation of neutral or type A **liposomes** containing zinc, 160 milligrams (mg) of hydrogenated **phosphatidylcholine** (HPC) or L-α-lecithin, (Avanti Polar Lipids, Birmingham, Ala.) were mixed with 40 mg of **cholesterol** (Chol), absolute ethanol (100 μL), and chloroform (100 μL) and incubated at 60° C. for 15 minutes. For preparation of exogenous materials to be entrapped in the **liposome**, a solution of 5 mL of aqueous buffer (e.g. Tris-saline buffer: 20 mM Tris, 0.15N NaCl, pH 7.4) containing the. . . 1 hour. The mixture was then cooled to 22° C. and centrifuged at 2000×g for 5 minutes to settle the **liposomes** to the bottom of the tube. The supernatant solution was removed after centrifugation, and the **liposomes** were washed in Tris-saline buffer, pH 7.4. After washing, the **liposomes** were again centrifuged and the wash supernatant was removed. The prepared **liposomes** were analyzed in a cell counter or particle analyzer to determine that their size (i.e. in terms of **liposome** volume) was in the range of about 4 to about 12 fL, with an mean of about 7 fL. The washed **liposome** zinc content was determined by x-ray fluorescence spectrometry. A **liposome** suspension, in which **liposomes** constituted 14% of the volume, resulted in a zinc value of 20.5 ppm zinc compared with the wash buffer control supernatant which

gave a value of around 5 ppm zinc. The prepared neutral **liposomes** were stored at 4° C. until use.

DETD Preparation of "Amine" or "Type B" **Liposomes**

DETD For the preparation of amine or type B **liposomes**, 160 milligrams (mg) of hydrogenated **phosphatidylcholine** (HPC) or lecithin, (Avanti Polar Lipids, Birmingham, Ala.) were mixed with 40 mg of **cholesterol** (Chol), and 2.7 mg of stearyl amine, absolute ethanol (100 μL), and chloroform (100 μL). The resulting mixture was incubated at 60° C. for 15 minutes. For preparation of exogenous materials to be entrapped in the **liposome**, a solution of 5 mL of aqueous buffer (e.g. 20 mM Tris, 0.15N NaCl, pH 7.4) containing the material to. . . 1 hour. The mixture was then cooled to 22° C. and centrifuged at 2000×g for 5 minutes to settle the **liposomes** to the bottom of the tube. The supernatant solution was removed after centrifugation and the **liposomes** were washed in Tris-saline buffer, pH 7.4. After washing, the **liposomes** were again centrifuged and the wash supernatant was removed. The prepared **liposomes** were analyzed in a cell counter or particle analyzer to determine that their size (i.e. in terms of **liposome** volume) was in the range of about 4 to about 12 fL, with an mean of about 7 fL. The prepared amine **liposomes** were stored at 4° C. until use.

DETD Preparation of Carboxylic Acid or "Type C" **Liposomes**

DETD For the preparation of carboxylic acid or type C **liposomes**, 160 milligrams (mg) of hydrogenated **phosphatidylcholine** (HPC) or lecithin, (Avanti Polar Lipids, Birmingham, Ala.) were mixed with 40 mg of **cholesterol** (Chol), and 2.7 mg of stearic acid, absolute ethanol (100 μL), and chloroform (100 μL). The resulting mixture was incubated at 60° C. for 15 minutes. For preparation of exogenous materials to be entrapped in the **liposome**, a solution of 5 mL of aqueous buffer (e.g. Tris-saline buffer: 20 mM Tris, 0.15N NaCl, pH 7.4) containing the. . . 60° C. for about 1 hour, cooled to 22° C., and centrifuged at 2000×g for 5 minutes to settle the **liposomes** to the bottom of the tube. The supernatant solution was removed after centrifugation and the **liposomes** were washed in Tris/saline pH 7.4 buffer. After washing, the **liposomes** were again centrifuged and the wash supernatant was removed. The prepared **liposomes** were analyzed in a cell counter or particle analyzer to determine that their size (i.e. in terms of **liposome** volume) was in the range of about 4 to about 12 fL, with an mean of about 7 fL. The prepared carboxylic acid **liposomes** were stored at 4° C. until use.

DETD Effects of **Liposomes** on the Breaking Strength, BS, (or Tensile Strength) of Fibrin Glue

DETD The breaking strength of the fibrin glue in the present fibrin glue and **liposome** composition was measured by mixing fibrin glue components in a plastic test tube and pipeting the still-liquid mixture into the. . .

DETD . . . determining the breaking or tensile strength of fibrin glue was useful for measuring the effects of the various types of **liposomes** on the mechanical properties of fibrin glue formed from cryoprecipitate or fibrin glue formed using pure fibrinogen (e.g., from Cohen Fraction I paste), (FIGS. 3, and 4). The results showed that a particular type of **liposome** was made and added to the fibrin glue components to form the fibrin glue composition at levels which did not significantly affect the mechanical properties and integrity of the fibrin glue formulation. Thus, **liposomes** can be added to the fibrin glue to produce the compositions of the invention in which the glue has a. . .

DETD Viscoelastic Effect Of **Liposomes** in Fibrin Glue

DETD . . . develops viscoelastic properties which can be monitored in a thromboelastograph as TEG amplitude. Tests with Type A, B and C **liposomes**, as described above, indicated that depending on the composition and proportions of components in the final fibrin glue composition, the **liposomes** may or may not significantly affect the viscoelasticity of the fibrin glue.

DETD . . . example, with low levels of thrombin (e.g. 0.5 U/mL final) mixed with fibrinogen from cryoprecipitate, Types A, B and C **liposomes** did not significantly increase the early phase of the development of

viscoelasticity. However, after 30 minutes, Type A and Type C **liposomes** increased the final viscoelasticity of the fibrin glue, while Type B **liposomes** had no significant effect (FIG. 5). The results indicate that the amine groups on the surfaces of Type B **liposomes** may diminish the mechanical properties of the fibrin glue, probably by interfering with the cross-linking of fibrin instigated by factor. . . amplitude was maximized within 2 minutes, and no significant difference could be detected among any of the fibrin glue and **liposome** formulations. These results indicate that **liposomes** with different surface moieties, such as the amine or carboxylic acid groups of the Type B and Type C **liposomes**, respectively, can be formulated, as desired, to affect the mechanical properties of fibrin glue as desired or needed in using the bioadhesive **liposome**-containing fibrin glue compositions.

DETD . . . concentration: 3.6 mg/mL; final volume: 300 uL) mixed with different volumes (either 50 μL or 100 uL) of Type A **liposome** suspensions. Clotting (i.e., gelation) was initiated by low levels of thrombin (e.g. 0.5 U/mL). Here, a small but significant increase of the TEG amplitude was observed with added **liposome** volume (FIG. 6). These results indicate that fibrin glue may be formulated by altering the volume of **liposomes** added in a manner which does not significantly interfere with the viscoelastic properties of the final fibrin glue and **liposome** composition.

DETD The ability of **liposomes** to modulate or not interfere with the viscoelastic properties of fibrin glue can be advantageous, such as when the fibrin glue and **liposome** composition is used to prepare films or membranes that need to remain flexible during use, or when the composition is. . . vivo for long periods of time. However, normal lyric processes could degrade the fibrin glue rather rapidly. For such uses, **liposomes** could be prepared with proteolytic inhibitors encapsulated within their aqueous compartments. With the onset of degradation of the glue, **liposomes** would be exposed and slowly release their entrapped proteolytic inhibitors. This process would thereby decrease the rate of degradation of the fibrin glue and **liposome** film or membrane. Thus, **liposomes** would minimally affect and even augment the desired mechanical properties of fibrin glue and would ultimately increase the effective lifetime. . .

DETD **Liposome** and fibrin glue composition used in wound healing of skin incisions

DETD To demonstrate the utility of **liposomes** entrapped in fibrin glue and used in animals, in vivo experiments were performed. For surgical incisions, a 2 cm longitudinal,. . . any blood from the field. The incision was either stapled or was sealed with fibrin glue without or with added **liposomes** which had been prepared to contain entrapped zinc as a type of bioadditive in accordance with the invention.

DETD Essentially, the Type A **liposomes** (i.e., neutral) were prepared by injecting a warmed solution of **cholesterol** and lecithin into Tris-saline buffer, pH 7.4, which contained 2 mM Zn(II) salt, and the resulting **liposomes** were incubated and washed as described. A Zn(lI) solution entrapped in **liposomes** was used as an exemplary bioadditive in the aqueous compartment of the **liposome**. One skilled in the art will appreciate that other bioadditives, and solutions containing such additives, are equally and particularly suitable for entrapment in the **liposomes** of the composition, as described in the Detailed Description of the Invention. The Zn(II)-loaded **liposomes** were analyzed by X-ray fluorescence and found to have encapsulated about 10-20% aqueous phase of their total volume. The Zn(II)-loaded **liposomes** were added to fibrinogen in cryoprecipitate (10% by volume) prior to mixing with thrombin at the site of incision, i.e., forming a fibrin glue and Zn(II)-loaded **liposome** matrix at the wound site.

DETD . . . heal and were sacrificed after 14 days. The skin incisions that had been sealed either with the fibrin glue and **liposome**-containing composition or with staples were excised and analyzed. The incision area was analyzed for the presence of zinc in the. . . wound that contained 6±2 ppm zinc as a normal background level, the wound tissue to which fibrin glue with zinc-entrapped **liposomes** had been applied,

contained double the zinc level (1.5%, 1022 ppm). The level of zinc found at the site of the stapled wound and at the site of application of the fibrin glue and zinc-containing **liposome** composition was compared with the level of zinc normally present in a non-cut region of the animal's skin or tissue. The results showed that a significantly increased level of zinc was released from **liposomes** relative to that of the normal controls and of normal undamaged skin.

DETD This experiment demonstrated that the **liposomes** delivered zinc (or other entrapped materials) to the tissue of an animal with a healing incision. These data showed that **liposomes** entrapped in fibrin glue delivered their encapsulated aqueous contents to a tissue site without interfering with the adhesion and sealing functions of the fibrin glue. Both the encapsulation of bioactive material in the **liposomes** and their fixation at a tissue site by fibrin glue were demonstrated to work in accordance with the invention as. . .

DETD . . . mg/ml) was placed in one syringe and 1 mL thrombin 0.5 U/ml, 2 mM Ca(II) and up to 200 μL **liposomes** were placed in a second syringe. The source of fibrinogen for preparation of the fibrin glue was from cryoprecipitate. As a control, fibrin glue was also formulated in the absence of **liposomes** and used to seal the incision. The contents of the syringes were released at the site of the incision to formulate the fibrin glue with or without added **liposomes** in situ. In other control animals, an incision was made as above, and the wound was closed with 4 surgical. . . WTS results obtained from control animals with stapled incisions, from control animals with wounds sealed with fibrin glue formulated without **liposomes**, and from experimental animals with wounds sealed with fibrin glue formulated with **liposomes** were compared (FIG. 7). These findings indicate that **liposomes** can augument the wound healing properties of fibrin glue.

DETD Films or Membranes Produced by Fibrin Glue and **Liposome** Compositions

DETD The addition of **liposomes** to fibrin glue can significantly and advantageously modify the physical characteristics of the film or membrane formed from the fibrin glue and **liposome** composition as described herein. For example, a 2 mm thick by 1 cm wide fibrin glue film made from fibrin glue containing 28 mg/mL fibrinogen, 10 U/mL thrombin, 15 mM Ca(II) solution, and **liposomes** (10% by volume) exhibited a breaking strength of 11 grams and became elongated by more than 200%. For convenience, the components used to formulate the fibrin glue and **liposome** composition were placed in an appropriate receptacle or container and sprayed onto the film or membrane; the spraying process mixed the components prior to their application to the substrate. After gelling or coagulation of the fibrin glue and **liposome** composition, the film or membrane of fibrin glue and **liposomes** was cleanly peeled away from the substrate film or membrane without the problems of sticking to the substrate or breakage during removal. For synthetic surfaces onto which the fibrin glue containing **liposome** film does adhere, the mechanism of adherence has not been completely elucidated, although ionic interactions are presumably involved. For films. . . mM Ca(II), an increase in film breaking strength is noted (e.g., to 18 g) (FIG. 8). In addition, Type A **liposomes** (i.e., neutral) and Type C **liposomes** (i.e., carboxylic acid) formulated in the fibrin glue composition increased the relative breaking strength and percent or degree of elongation, while Type B **liposomes** (i.e., amine) decreased the relative breaking strength and the percent of elongation before breaking (FIGS. 8 and 9). This example demonstrates that **liposomes** incorporated into fibrin glue films can modulate its physical parameters in a controlled manner, and also indicates that films to be used as wound dressings or membrane devices can be fabricated from fibrin glue films which contain **liposomes**.

DETD Fibrin Glue and **Liposome** Composition for Sealing Bone Breaks

DETD To illustrate the technique of using the fibrin glue containing **liposome** composition to seal and repair bone breaks, 50 mg of sheep femur bone fragments (not longer than 2 mM) were. . . mixed with fibrin glue composed of 1 mL of 50 mg/mL fibrinogen without or with 5% Coy volume) Type A **liposomes**, 300 uL of thrombin (10 U/mL), and 50 mM Ca(II). The fibrin glue, **liposomes**, and bone matrix was allowed to set

techniques described above. The results indicated that Type A **liposomes** did not significantly decrease the mechanical properties of the fibrin glue and **liposome** composition which had been admixed with bone fragments (see FIG. 12).

CLM What is claimed is:

1. A bioadhesive composition comprising fibrin glue and **liposomes**, said composition comprising: (i) about 10 to 90 mg/mL fibrinogen; (ii) about 1 to 200 U/mL thrombin; and (iii) about 1 to 30 mM calcium; in admixture with about 1% to 20%, by volume, **liposomes**, said **liposomes** embedded in said glue after clotting of said glue so as to localize said **liposomes** within a site of administration of said fibrin glue and **liposome** bioadhesive composition.

2. A **liposome**-containing fibrin glue composition comprising about 1% to 20%, by volume, **liposomes** combined with about 10 to 90 mg/mL fibrinogen, about 1 to 200 U/mL thrombin, and about 1 to 30 mM calcium to form a fibrin glue bioadhesive clot, said **liposomes** sequestered and embedded within said fibrin glue clot.

. . . 2, wherein said composition comprises fibrinogen at a concentration of 30-60 mg/mL; thrombin at a concentration of about 5-100 U/mL; **liposomes** at about 5-10% by volume; and calcium at a concentration of about 10-15 mM.

. . . claim 3, wherein said composition comprises fibrinogen at a concentration of 40 mg/mL; thrombin at a concentration of 10 U/mL; **liposomes** at about 10%, by volume, and calcium at about 10 mM.

. . . claim 1 or claim 2, wherein one or more biologically active ingredients is contained within the aqueous phase of said **liposomes**.

15. The composition according to claim 8, wherein said **liposomes** are neutral **unilamellar** vesicles.

16. The composition according to claim 8, wherein said **liposomes** are charged **unilamellar** vesicles.

17. The composition according to claim 8, wherein said **liposomes** comprise a mixture of saturated and unsaturated lipids.

. . . claim 1 or 2, wherein one biologically active ingredient is contained within the aqueous compartment of a first set of **liposomes** selected from the group consisting of **liposomes** formulated to contain noncharged chemical groups exposed on the **liposome** surface, thereby resulting in neutral **liposomes**; **liposomes** formulated to contain amine groups exposed on the **liposome** surface, thereby resulting in amine **liposomes**; and **liposomes** formulated to contain carboxylic acid groups exposed on the **liposome** surface, thereby resulting in carboxylic acid **liposomes**, and another biologically active ingredient is contained within the aqueous compartment of a second set of **liposomes** selected from the group consisting of **liposomes** formulated to contain noncharged chemical groups exposed on the **liposome** surface, thereby resulting in neutral **liposomes**; **liposomes** formulated to contain amine groups exposed on the **liposome** surface, thereby resulting in amine **liposomes**; and **liposomes** formulated to contain carboxylic acid groups exposed on the **liposome** surface, thereby resulting in carboxylic acid **liposomes**, said first and second sets of **liposomes** being mixed and sequestered in the fibrin glue to form said composition, wherein the biologically active ingredients are released.

20. The composition according to claim 18, wherein said first set of **liposomes** comprises neutral **liposomes** formulated to contain noncharged chemical groups exposed on their surfaces, and said second set of **liposomes** comprises neutral **liposomes** formulated to contain noncharged chemical groups exposed on their surfaces.

21. The composition according to claim 18, wherein said first set of **liposomes** comprises neutral **liposomes** formulated to contain noncharged chemical groups exposed on their surfaces and said second set of **liposomes** comprises **liposomes** formulated to contain amine groups exposed on their surfaces.

22. The composition according to claim 18, wherein said first set of **liposomes** comprises neutral **liposomes** formulated to contain noncharged chemical groups exposed on their surfaces and said second set of **liposomes** comprises **liposomes** formulated to contain carboxylic acid groups exposed on their surfaces.

23. The composition according to claim 18, wherein said first set of **liposomes** comprises **liposomes** formulated to contain amine groups exposed on their surfaces and said second set of **liposomes** comprises **liposomes** formulated to contain carboxylic acid groups exposed on their surfaces.

24. The composition according to claims 1 or 2, wherein said **liposomes** are formulated to contain on their surfaces compounds having at least one light sensitive chemical double bond, said double bond predisposing the compounds to undergo conformational change when exposed to light, thereby resulting in photoactivable or light sensitive **liposomes**.

25. The composition according to claim 1 or 2, wherein said composition comprises about 1% to about 15% **liposomes** by volume.

26. The composition according to claim 25, wherein said composition comprises about 5% to about 10% **liposomes** by volume.

27. The composition according to claim 26, wherein said composition comprises about 10% **liposomes**.

28. The composition according to claim 1 or claim 2, wherein said composition is applied as a liquid or spray or paste to form a fibrin glue-containing **liposome** film or membrane.

. . . to claim 2, wherein said composition is applied as a liquid or spray ex vivo to form a fibrin glue-containing **liposome** foam or gel.

33. The composition according to claim 28 or claim 30, wherein **liposomes** are present at about 1% to about 15% by volume.

34. The composition according to claim 33, wherein said **liposomes** are present at about 10% by volume.

35. The composition according to claim 17, wherein said **liposomes** are formulated to contain noncharged or neutral lipids exposed on their surface membranes, thereby resulting in neutral **liposomes**.

36. The composition according to claim 35, wherein said noncharged or neutral lipids are lecithin or **phosphatidylcholine**.

37. The composition according to claim 17, wherein said **liposomes** are formulated to contain amine groups exposed on the **liposome** surface, thereby resulting in amine **liposomes**.

39. The composition according to claim 17, wherein said **liposomes** are formulated to contain carboxlic acid groups exposed on the **liposome** surface, thereby resulting in carboxylic acid **liposomes**.

41. The composition according to claim 21, wherein said first set of neutral or noncharged **liposomes** is formulated to contain **phosphatidylcholine** or lecithin exposed on the **liposome** surface and said second set of amine **liposomes** is formulated to contain stearyl

amine or diethylstearylamine exposed on the **liposome** surface.

42. The composition according to claim 41, wherein said first set of neutral or noncharged **liposomes** is formulated to contain **phosphatidylcholine** or lecithin exposed on the **liposome** surface and said second set of carboxylic acid **liposomes** is formulated to contain stearic acid exposed on the **liposome** surface.

43. The composition according to claim 23, wherein said first set of amine **liposomes** is formulated to contain stearyl amine or diethylstearylamine exposed on the **liposome** surface and said second set of carboxylic acid **liposomes** is formulated to contain stearic acid exposed on the **liposome** surface.

L17 ANSWER 13 OF 15 USPATFULL on STN
96:120605 **Liposome** dispersion.
Moynihan, Karen L., Brea, CA, United States
NeXstar Pharmaceuticals, Inc., Boulder, CO, United States (U.S. corporation)
US 5589189 19961231
APPLICATION: US 1994-306036 19940914 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A process for the production of a non-aggregating, filterable dispersion of liposomal encapsulated hemoglobin includes the addition of a plasma protein such as human serum albumin to a **liposome** dispersion and the application of **liposome**-forming energy to the dispersion sufficient to form **unilamellar** vesicles having a diameter smaller than 0.2 μm.

TI **Liposome** dispersion

AB . . . filterable dispersion of liposomal encapsulated hemoglobin includes the addition of a plasma protein such as human serum albumin to a **liposome** dispersion and the application of **liposome**-forming energy to the dispersion sufficient to form **unilamellar** vesicles having a diameter smaller than 0.2 μm.

SUMM . . . blood types to avoid antigenic response, and also presents the possibility of contamination by blood born pathogens such as the **human immunodeficiency virus**. Thus, a search for blood substitutes has been undertaken in order to provide an alternative to blood transfusion.

SUMM . . . of cell-free hemoglobin solutions, many have sought to create a synthetic red blood cell with hemoglobin encapsulated therein. For example, **liposome** encapsulated hemoglobin formulations and methods for making them are known in the art: U.S. Pat. Nos. 4,776,991 and 4,911,929 (Farmer),. . .

SUMM **Liposomes** are microscopic vesicles made from phospholipids, which form closed, fluid filled spheres when dispersed with aqueous solutions. Phospholipid molecules are. . . polar heads point in opposite directions toward an aqueous medium. These bilayer membranes thus form closed, hollow spheres known as **liposomes**. The polar heads at the inner surface of the membrane point toward the aqueous interior of the **liposome** and, at the opposite surface of the spherical membrane, the polar heads interact with the surrounding aqueous medium. As the **liposomes** are formed, water soluble molecules can be incorporated into the aqueous interior, and lipophilic molecules may be incorporated into the lipid bilayer. **Liposomes** may be either multilamellar, like an onion with liquid separating many lipid bilayers, or **unilamellar**, with a single bilayer surrounding an aqueous center.

SUMM Methods for producing **liposomes** are well known in the art, and there are many types of **liposome** preparation techniques which may be employed to produce various types of **liposomes**. These can be selected depending on the use, the chemical intended to be entrapped, and the type of lipids used to form the bilayer membrane. The requirements which must be considered in producing a **liposome** preparation are similar to those of other controlled release mechanisms. They are: (1) a high percent of chemical entrapment; (2). . .

SUMM The first method described to encapsulate drugs or other chemicals in **liposomes** involved the production of multilamellar vesicles (MLVs). **Liposomes** can also be formed as **unilamellar** vesicles (UVs), which

generally have a size less than 0.5 μm (μm, also referred to as "microns"). There are several techniques known in the art which are used to produce **unilamellar liposomes**.

SUMM Smaller **unilamellar** vesicles can be formed using a variety of techniques, such as applying a force sufficient to reduce the size of the **liposomes** and or produce smaller **unilamellar** vesicles. Such force can be produced by a variety of methods, including homogenization, sonication or extrusion (through filters) of MLVs.. . . diameters of up to 0.2 μm, which appear as clear or translucent suspensions. Other standard methods for the formation of **liposomes** are known in the art, for example, methods for the commercial production of **liposomes** include the homogenization procedure described in U.S. Pat. No. 4,753,788 to Gamble, a preferred technique, and the method described in. . .

SUMM Another method of making **unilamellar** vesicles is to dissolve phospholipids in ethanol and inject them into a buffer, whereby the lipids will spontaneously rearrange into **unilamellar** vesicles. This provides a simple method to produce UVs which have internal volumes similar to that of those produced by. . .

SUMM The therapeutic uses of **liposomes** include the delivery of drugs which are normally toxic in the free form. In the liposomal form the toxic drug may be directed away from the sensitive tissue and targeted to selected areas. **Liposomes** can also be used therapeutically to release drugs, over a prolonged period of time, reducing the frequency of administration. In addition, **liposomes** can provide a method for forming an aqueous dispersion of hydrophobic chugs for intravenous delivery.

SUMM When **liposomes** are used to target encapsulated drugs to selected host tissues, and away from sensitive tissues, several techniques can be employed. These procedures involve manipulating the size of the **liposomes**, their net surface charge as well as the route of administration. More specific manipulations have included labeling the **liposomes** with receptors or antibodies for particular sites in the body. The route of delivery of **liposomes** can also affect their distribution in the body. Passive delivery of **liposomes** involves the use of various routes of administration, e.g., intravenous, subcutaneous and topical. Each route produces differences in localization of the **liposomes**. Two methods used to actively direct the **liposomes** to selected target areas are binding either antibodies or specific receptor ligands to the surface of the **liposomes**. Antibodies are known to have a high specificity for their corresponding antigen and have been shown to be capable of being bound to the surface of **liposomes**, thus increasing the target specificity of the **liposome** encapsulated drug.

SUMM Since the chemical composition of many drugs precludes their intravenous administration, **liposomes** can be very useful in adapting these drugs for intravenous delivery. Furthermore, since **liposomes** are essentially hollow spheres made up of amphipathic molecules, they can entrap hydrophilic drugs in their aqueous interior space and hydrophobic molecules in their lipid bilayer. Unwanted molecules that remain in the dispersion external to the **liposomes**, such as unentrapped agents, are removed by column chromatography or ultrafiltration. Although methods for making **liposomes** are well known in the art; it is not always possible to determine a working formulation without experimentation.

SUMM . . . generation of a sterile parenteral product, and has been found to be the most effective in terms of processing and **liposome** stability. The best method for terminal sterile filtration is the sequential filtration of a dispersion of **liposomes** through a 0.45 and 0.22 micron filtration system, and **liposomes** larger than 0.2 μm or aggregations of smaller **liposomes** will obstruct and clog this filter system, as well as the ultrafiltration system employed to remove unentrapped components. The Farmer patents disclose the small scale filtration of a **liposome** encapsulated hemoglobin formulation dispersed in a hyperosmotic buffered saline solution through a 0.22 micron filter. Similarly, Djordjevich discloses a laboratory process for filtering **liposome** encapsulated hemoglobin dispersed in a saline solution through a 0.22 micron filter for purposes of sterilization.

SUMM another important aspect of developing a **liposome** formulation is to achieve a unimodal or controlled particle size distribution of **unilamellar liposomes** having a median size less then 0.2 μm. Controlling the particle size distribution provides not only for a sterile filterable. . .

SUMM It has been desideratum to provide a process for preparing dispersion of hemoglobin encapsulating **unilamellar liposomes** that have a unimodal size distribution that do not aggregate, thus are capable of being ultrafiltered and are sterile filterable.

SUMM According to the invention, a method is provided for producing a non-aggregating, filterable dispersion of **liposomes** comprising forming a solution containing a dispersion of multilamellar **liposomes** and subjecting the solution to a force sufficient to reduce the size of the lipsomes; and adding a globular, preferably. . . of the force. The protein may be added after the beginning of the application of the force, provided that the **liposomes** are subjected to a further force sufficient to produce a dispersion of **unilamellar liposomes**. The protein may also be added prior to the initial application of the **liposome** forming force, provided that the solution to which the protein is added is a low ionic strength aqueous solution or. . . is essentially free of ions (having essentially no ionic strength), and particularly being essentially free of phosphate ions, during the **liposome** forming procedure, e.g., during homogenization.

SUMM The **liposome** forming force utilized must be sufficient to produce **unilamellar** vesicles having a mean diameter of less than 0.2 μm. The resulting **liposomes** produced have a particle size distribution wherein 85 to 100 percent, by volume, of the **liposomes** have a median diameter of less than 0.2 μm. The preferred method used to provide a force to reduce the. . . globulin; α-1 lipoprotein and mixtures thereof. The preferred protein is human serum albumin. A preferred formulation includes incorporating into the **liposomes** an active agent such as a therapeutic or an imaging agent. The preferred size range of the **liposomes** is between 0.08 and 0.15 μm, with the median diameter.

SUMM Also provided is a process for producing **liposome** encapsulated hemoglobin comprising: forming a solution including a plasma protein and a dispersion of **liposome** encapsulated hemoglobin; subjecting the solution to a force sufficient to reduce the median size of the **liposomes** to less then 0.2 μm wherein the resulting **liposomes** are **unilamellar** and have a unimodal particle size distribution, provided that when the protein is added prior to the application of the. . . the solution comprises an aqueous solution containing less then or equal to 20-40 mM phosphate buffer; and filtering the resulting **liposomes** through a filter passing particles having a size less then or equal to 0.45 microns. The **liposomes** can be ultra- or sterile filtered in the absence of a hyperosmotic buffered saline and in the presence of sucrose. The process also results in a **liposome** formulation that is suitable for lyophilization.

SUMM Also provided is a process for producing **liposomes**, that includes forming a solution including a plasma protein and a dispersion of **liposomes**; then subjecting the solution to a force sufficient to reduce the median size of the **liposomes** to less then 0.2 μm wherein the resulting **liposomes** are **unilamellar** and have a unimodal particle size distribution; and filtering the resulting **liposomes** through a filter having a size less then or equal to 0.45 microns. More specifically, a process is provided for producing a **liposome** encapsulated hemoglobin comprising: (a) forming a solution including a plasma protein and a dispersion of **liposome** encapsulated hemoglobin; and then (b) subjecting the solution to a force sufficient to reduce the median size of the **liposomes** to less then 0.2 μm wherein the resulting **liposomes** are **unilamellar** and have a unimodal particle size distribution.

SUMM . . . preferred process of the present invention is initiated with the formation of a powder or film containing a neutral lipid, **cholesterol**, and a negatively charged lipid. Alpha tocopherol is a preferred ingredient. Neutral lipids suitable for use include egg

phosphatidylcholine, distearoyl **phosphatidylcholine** (DSPC), hydrogenated soy choline (HSPC), dimyristoylphosphatidylcholine (DMPC), hydrogenated egg **phosphatidylcholine** (HEPC), and dipalmitoylphosphatidylcholine. The preferred neutral lipids have carbon chain lengths from C_{16} -C_{18}. The preferred neutral lipid is DSPC.. . . lipid is DMPG. The lipid film or powder is provided containing a 100 mg/ml of lipid wherein the formulation contains DSPC:**cholesterol**:DMPG:α-tocopherol in a molar ratio of 10:9:1:0.4. This general formula is taught in U.S. Pat. No. 4,911,929 to Farmer. It is. . . lipid is approximately 50% of the total lipid; the negatively charged lipid is approximately 10% of the total lipid and **cholesterol** is approximately 40% of the total lipid; and the α tocopherol is approximately 2% of the total lipid.

SUMM . . . end with the use of a 0.45 micron filter and 0.2 micron filter to provide for a sterile solution of **liposome** encapsulated hemoglobin suitable for liquid storage at 4° or lyophilization.

SUMM . . . present invention, other sources of hemoglobin including recombinant hemoglobin or bovine/porcine hemoglobin may also be used. This procedure results in **liposome** encapsulated hemoglobin having a median size of 0.09 to 0.15 μm wherein the particle size distribution is normally displayed having. . . the initial application of force. In all cases a second application of energy sufficient to reduce the size of the **liposomes** is necessary once the albumin is added to the solution. The preferred buffer solution contains less then 20 mM phosphate.. . . a hydrophobic pocket, might be able to "coat" sites of unencapsulated or denatured hemoglobin residing on the surface of the **liposomes** when added in conjunction with the application of a force necessary to reduce the size of the **liposome**, and thus disrupt the tendency toward aggregation. The concepts noted herein are also suitable for other formulations having a surface. . .

SUMM . . . Previous studies in our laboratories have shown that without the addition of a protein such as albumin as disclosed herein, **liposome** encapsulated hemoglobin dispersions irreversibly aggregate. The aggregation after sonication or homogenization was characterized by a particle size distribution having a broad multimodal distribution with two main size populations. The population of **liposomes** having a median size less than 0.2 μm was generally less than 60 percent by volume as measured by laser. . . size and a second population was approximately 1 to 2 microns in median diameter. FIG. 1 is illustrative of a **liposome** encapsulated hemoglobin formulation homogenized in the absence of saline and in the presence of a 9% sucrose solution. It was. . .

SUMM The use of albumin in **liposome** formulations for other purposes is known. For example, ox albumin is added to a colloidal dispersion of **liposomes** as a stabilizer during dehydration in U.S. Pat. No. 4,229,360. Proteins, and in particular bovine albumin, have been disclosed as suitable for use as a cryoprotectant in WO 90/03795. U.S. Pat. No. 4,746,516 discloses the lyophilization of **liposomes** in the presence of hydrophilic excipients, including albumin.

SUMM The general idea of the use of albumin, a physiologically acceptable excipient, to prevent aggregation in **liposome** dispersions was first investigated by the addition of albumin after **liposome** formation (that is, after the application of the **liposome** forming force) and before or during filtration. Albumin was introduced into a solution containing a dispersion of **liposome** encapsulated hemoglobin via a dialysis/ultrafiltration system at two concentrations (1% and 5%). The ultrafiltration step initially commenced at 10% albumin. . . during processing of the 10% lot forced dilution to 1%. The addition of albumin to the external phase of the **liposomes** did not result in the prevention of post homogenization aggregation.

DETD **Liposome** encapsulated hemoglobin samples were prepared as follows. Distearoyl **phosphatidylcholine**, **cholesterol**, dimyristoylphosphatidylglycerol, and α-tocopherol in a molar ration of 10:9:1:0.4 were dissolved in chloroform and methanol (1:1 by volume). The resulting. . .

DETD . . . post pass 15 and post pass 30 with deionization. The post hydration sample sonicated with albumin contained only 19% of **liposome** particles in the desired range of approximately 100 nm, suggesting that at this phosphate concentration, albumin added prior to the application of the force sufficient to reduce the size of **liposomes** did not decrease aggregation. In fact, this sample had a less deskable particle size distribution than samples prepared in the. . .

DETD The samples probe sonicated in the presence of albumin after they were passed through the homogenizer increased the populations of **liposomes** in the approximately 100 nm range peak to 82% and 85% as shown in Tables 2 and 3. The samples. . . (PVDF filters) series (the particle size of LEH was generally equal to 113.0 nm median diameter post filtration). The resulting **liposome** encapsulated hemoglobin was purified from unencapsulated hemoglobin on a Sephacryl S-300 column eluted with 9% sucrose and 30 mM phosphate, pH 7.4. The **liposome** fraction was collected and filtered through a tandem series of PVDF filters 0.8, 0.45 and 0.22 microns yielding a preparation. . .

DETD **Liposomes** were prepared as in Example 1 except that they were hydrated in either a 9% sucrose, 30 mM pH 7.4. . . obtained for the zero phosphate samples prepared and removed after one pass, when albumin is added, displayed a majority of **liposomes** (mean volume greater than 90%), having a median size of from 90 nm to 121 nm. Most of these samples. . . serum of albumin each time followed by brief probe sonication. Samples removed at pass 1, 15, or 30 all yielded **liposome** encapsulated hemoglobin with particle size distribution of 90 to 95 nm median diameter with practically all samples displaying a unimodal. . .

DETD This experiment was conducted to determine the effect of buffer strength in producing sterile filterable **liposomes** having a unimodal particle size distribution when albumin is added prior to the addition of any force sufficient to reduce the size of the **liposomes**. Multilamellar **liposomes** were obtained as in example 1 just after hydration) with various phosphate buffer strength and quantity of human serum albumin. . . particle size was measured. Table 4 summarizes the results. As can be seen at 40 mM phosphate buffer and below, **liposome** encapsulated hemoglobin dispersed in a solution is formed having a size distribution which is suitable for sterile filtering. The significance. . . may be added to the solution prior to the application of a force sufficient to reduce the size of the **liposome**.

DETD The following experiment was conducted to identify plasma proteins other than albumin that are suitable to yield non-aggregated filterable **liposome** encapsulated hemoglobin. A lipid solution was hydrated as in example 1 in a pH 7.4 0 mM aqueous solution (9%. . .

DETD . . . 30 minutes at 0° to 5° C., in 9% sucrose hemoglobin solution with no phosphate. The resulting multilamellar solution of **liposomes** was then homogenized at 4000 psi, at 20-25 degrees celsius for 12-16 passes. The solution was then ultrafiltered to recover. . .

DETD . . . assess the potential antigenicity of albumin as used in the process. Female Balb-c mice were injected with a solution of **liposome** encapsulated hemoglobin prepared with albumin and compared to empty (no hemoglobin) **liposome** encapsulated hemoglobin prepared with albumin and 9% sucrose with albumin. No toxicities were noted or deaths encountered in any of. . .

CLM What is claimed is:

1. A process for producing a non-aggregating dispersion of **liposomes**, comprising: a) forming a dispersion of preformed **liposomes** containing an active therapeutic or an imaging agent; b) adding a solution of a globular protein to the dispersion; c) then subjecting the dispersion to a force sufficient to reduce the median size of the **liposomes** to less than 0.2 μm wherein the resulting **liposomes** are **unilamellar**, provided that when the globular protein is added prior to an initial application of force the dispersion comprises an aqueous solution having an ionic strength of below 50 mM; and d) filtering the resulting **liposomes** through a filter having a size less than or equal to 0.45 μm, wherein the protein is selected from the. . .

3. The process of claim 1 wherein the **liposomes** have a median diameter of less that 0.15 μm.

4. The process of claim 2 wherein the **liposomes** have a median diameter of less that 0.15 μm.

L17 ANSWER 14 OF 15 USPATFULL on STN
94:9574 Methods of treating cancer using modified C-reactive protein.
Potempa, Lawrence A., Deerfield, IL, United States
Kresl, John J., Evanston, IL, United States
Anderson, Byron E., Morton Grove, IL, United States
Immtech International, Inc., Evanston, IL, United States (U.S. corporation)Northwestern University, Evanston, IL, United States (U.S. corporation)
US 5283238 19940201
APPLICATION: US 1992-874263 19920424 (7)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The invention provides a method of treating cancer in a mammal comprising administering to the mammal an effective amount of modified C-reactive protein ("mCRP"). The invention also provides a method of treating cancer in a mammal comprising administering to the mammal mCRP in combination with another agent such as a chemotherapeutic compound, immunoadjuvant, or cytokine. The mCRP may be administered to the mammal in a pharmaceutically-acceptable carrier or in **liposomes**. The invention further provides a method of identifying cancer cells in a mammal using mCRP as an imaging agent.

AB . . . a chemotherapeutic compound, immunoadjuvant, or cytokine. The mCRP may be administered to the mammal in a pharmaceutically-acceptable carrier or in **liposomes**. The invention further provides a method of identifying cancer cells in a mammal using mCRP as an imaging agent.

SUMM . . . of U.S. application 07/176,923, filed Apr. 4, 1988, now abandoned. Further, mCRP is useful in treating viral infections such as **human immunodeficiency virus** 1 ("**HIV**-1"), as disclosed in co-pending U.S. application Ser. No. 07/799,448, filed Nov. 27, 1991. Finally, mCRP can be used in treating. . .

SUMM The invention also provides a method of treating cancer in a mammal comprising administering to the mammal a plurality of **liposomes** collectively containing an effective amount of modified-CRP.

DETD Alternatively, a plurality of **liposomes** collectively containing an effective amount of mCRP may be administered to the mammal. The term "**liposome**" in the present invention relates to any sac-like or hollow vesicle-like structure which is capable of encapsulating mCRP, and includes but is not limited to, multilamellar vesicles, **unilamellar** vesicles, and red blood cell ghosts. Methods of preparing **liposomes** and encapsulating molecules in **liposomes** are well known in the art.

DETD Preferably, the **liposomes** containing mCRP are **unilamellar** vesicles formed by extrusion, referred to herein as "LUVETs." Methods of preparing LUVETs are described by MacDonald et al., Biochim.. . . particularly useful for administering mCRP because LUVETs do not contain traces of organic solvents and detergents commonly used in preparing **liposomes**. Also, LUVETs are defined by a single lipid bilayer, contain relatively large internal volumes, and typically have greater encapsulation efficiency as compared to multilamellar vesicles. Although the exact mechanism(s) by which **liposomes** containing mCRP function is not fully understood, it is believed that the **liposomes** may act as a vehicle for efficient delivery of mCRP to the cancer cells.

DETD . . . mg per kilogram, is effective for treating cancer, whether the mCRP is administered in a pharmaceutically-acceptable carrier or contained in **liposomes**. It is understood by those skilled in the art that the dose of mCRP that must be administered will vary. . .

DETD . . . mammal in combination with another agent. The mCRP may be administered to the mammal in a pharmaceutically-acceptable carrier or in **liposomes**, as described above. The agent administered in combination with the mCRP may be a naturally-occurring or a synthetic

substances, and. . .

DETD . . . two previously described methods--a first method that utilized mild conditions for the entrapment of biologically active macromolecules [Kirby et al., **Liposome** Technology, Volume I, Preparation of **Liposomes**, CRC Press, pp. 19-27 (1984)] and a second method to form vesicles of well defined size and homogeneity [Olson et. . .

DETD A mixture of L-α-lecithin (**phosphatidylcholine**), sphingomyelin, (Avanti Polar Lipids, Inc., Pelham, Ala.) and **cholesterol** (Sigma) was placed in a round bottom flask in a molar ratio of 1:1:1 and dissolved in HPLC grade chloroform. . .

DETD TABLE 1

	Multilamellar Vesicles (MLVs)	Large **Unilamellar** Vesicles (LUVETS)
Amount protein/ lipid	0.28 μg/μmol lipid	2.0 μg/μmol lipid
Internalization	0.82 μl/μmol lipid	2.02 μl/μmol lipid
Volume Encapsulation	341.5 ± 53 μg. . .	

DETD . . . CRP, is an effective therapy in preventing the growth of murine breast adenocarcinoma. Furthermore, mCRP is effective whether injected in **liposomes** or alone in buffer.

CLM What is claimed is:

3. A method of treating adenocarcinoma in a mammal comprising administering to the mammal a plurality of **liposomes** collectively containing an effective amount of modified-CRP.

4. The method of claim 3 wherein the **liposomes** are **unilamellar** vesicles.

5. The method of claim 4 wherein the **unilamellar** vesicles are LUVETs.

8. A method of inhibiting adenocarcinoma metastasis in a mammal comprising administering to the mammal a plurality of **liposomes** collectively containing an effective amount of modified-CRP.

9. The method of claim 8 wherein the **liposomes** are **unilamellar** vesicles.

10. The method of claim 9 wherein the **unilamellar** vesicles are LUVETs.

L17 ANSWER 15 OF 15 USPATFULL on STN

92:25125 Steroidal **liposomes** exhibiting enhanced stability.

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CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to novel **liposomes** and **liposome**-like structures (vesicles) comprising an amount of a derivatized sterol either alone or in combination with additional **liposome**-forming lipids.

Sterols such as **cholesterol** or other lipids, to which numerous charged or neutral groups are attached, may be used to prepare **liposomes** and **liposome**-like structures such as micelles, reverse micelles and

hexagonal phases, suspensions of multilamellar vesicles or small **unilamellar** vesicles. The novel **liposomes** of the present invention may be prepared with or without the use of organic solvents. These vesicles may entrap compounds varying in polarity and solubility in water and other solvents. The vesicles of the present invention may function as vaccines after entrapment or association of an immunogen, as adjuvants, either alone or in combination with additional adjuvants, including, for example, Freund's adjuvant (and other oil emulsions), Bortedella Pertussis, aluminum salts and other metal salts and Mycobacterial products (including muramyldipeptides), among others. The present invention relates to novel **liposomes** and **liposome**-like structures (vesicles) comprising an amount of a derivatized sterol either alone or in combination with additional **liposome**-forming lipids.

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PARN . . . "Vaccine Steroidal Adjuvant", filed Aug. 25, 1988, now abandoned, and a continuation-in-part of U.S. patent application Ser. No. 236,701, entitled "DMPC/**Cholesterol** Adjuvant, filed Aug. 25, 1988, now abandoned.

SUMM The present invention relates to novel **liposomes** and **liposome**-like structures (vesicles) comprising an amount of a derivatized sterol either alone or in combination with additional **liposome**-forming lipids.

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SUMM **Liposomes** are completely closed lipid bilayer membranes which contain entrapped aqueous volume. **Liposomes** are vesicles which may be **unilamellar** (single membrane) or multilamellar (onion-like structures characterized by multiple membrane bilayers, each separated from the next by an aqueous layer).. . . toward the center of the bilayer, whereas the hydrophilic (polar) "heads" orient toward the aqueous phase. The basic structure of **liposomes** may be made by a variety of techniques known in the art.

SUMM The original **liposome** preparation of Bangham, et al. (J. Mol. Biol., 1965, 13:238-252) involves suspending phospholipids in an organic solvent which is then. . . reaction vessel. Next, an appropriate amount of aqueous phase is added, the mixture is allowed to "swell" and the resulting **liposomes** which consist of multilamellar vesicles (MLVs) are dispersed by mechanical means. This technique provides the basis for the development of the sonicated **unilamellar** vesicles described by Papahadjopoulos et al. (Biochim. Biophys. Acta., 1968, 135:624-638), and large **unilamellar** vesicles. Small **unilamellar** vesicles have a diameter of about 100 nm or less.

SUMM **Unilamellar** vesicles may be produced using an extrusion apparatus by a method described in Cullis et al., PCT Application No. WO 86/,00238, published Jan. 16, 1986, entitled "Extrusion Technique for Producing **Unilamellar** Vesicles" incorporated herein by reference. Vesicles made by this technique, called LUVETS, are extruded under pressure once or a

be included in the term "**unilamellar** vesicle".

SUMM Another class of multilamellar **liposomes** are those characterized as having substantially equal lamellar solute distribution. This class of **liposomes** is denominated as stable plurilamellar vesicles (SPLV) as defined in U.S. Pat. No. 4,522,803 to Lenk, et al., monophasic vesicles. . . . thaw cycle. The FATMLV procedure is described in Bally et al., PCT Publication No. 87/00043, Jan. 15, 1987, entitled "Multilamellar **Liposomes** Having Improved Trapping Efficiencies", corresponding to U.S. Pat. No. 4,975,282, issued Dec. 4, 1990. U.S. Pat. No. 4,721,612to Janoff et al. describes steroidal **liposomes** for a variety of uses. The teachings of these references as to preparation and use of **liposomes** are incorporated herein by reference.

SUMM . . . an immunogen that is followed by one or more booster exposures to the immunogen. Priming with relatively strong immunogens and **liposomes** is discussed in "Liposomal Enhancement of the Immunogenicity of Adenovirus Type 5 Hexon and Fiber Vaccines", Kramp, W. J. et al., Infection and Immunity, 25:771-773 (1979) and "**Liposomes** as Adjuvants with Immunopurified Tetanus Toxoid: the Immune Response", Davis, D. et al., Immunology Letters, 14:341-8 (1986/1987).

SUMM . . . Freund's Adjuvants (and other oil emulsions), Bortedella Pertussis, aluminum salts (and other metal salts), Mycobacterial products (including muramyl dipeptides), and **liposomes**. As used herein the term "adjuvant" will be understood to mean a substance or material administered together or in conjunction. . . immunogen. Adjuvants may be in a number of forms including emulsion (e.g., Freund's adjuvant) gels (aluminum hydroxide gel) and particles (**liposomes**) or as a solid material.

SUMM In particular embodiments adjuvants are comprised of **liposomes**. U.S. Pat. No. 4,053,585 issued Oct. 17, 1977 to Allison et al. states that **liposomes** of a particular charge are adjuvants. Davis, D, et al., "**Liposomes** as Adjuvants with Immunopurified Tetanus Toxoid: Influence of Liposomal Characteristics", Immunology, 61:229-234 (1987) and; Gregoriadis, G. et al., "**Liposomes** as Immunological Adjuvants: Antigen Incorporation Studies", Vaccine, 5:145-151 (1987) report DMPC/**cholesterol liposomes** (1:1) and immunogen as giving minimally improved (over free immunogen) immunological response in **unilamellar** vesicles of a distinct dehydration/rehydration type with tetanus toxoid as the immunogen, a strong immunogen. In the Davis and in. . .

SUMM . . . soluble derivatives have been used in cosmetics, pharmaceuticals and diagnostics. Of the water soluble sterols, for example, branched fatty acid **cholesterol** esters, steroid esters and PEG-phytosterols have been used in cosmetic preparations (U.S. Pat. No. 4,393,044 and European Patent Application No.. . . p. 46). A number of water soluble cholesterols have been prepared and used as water-soluble standards for the determination of **cholesterol** levels in biological fluids (See, for example, U.S. Pat. Nos. 3,859,047 4,040,784; 4,042,330; 4,183,847; 4,189,400; and 4,224,229). Shinitzky et al.. . . (1979, Proc. Natl. Acad. Sci. USA, 76, 5313) incubated tumor cells in tissue culture medium containing a low concentration of **cholesterol** and cholesteryl hemisuccinate. Incorporation of **cholesterol** or cholesteryl hemisuccinate into the cell membrane decreased membrane fluidity and increased membrane-lipid microviscosity.

SUMM **Cholesterol** and other sterols, have also been incorporated into phospholipid **liposome** membranes in order to alter the physical characteristics of the lipid bilayers. For example, Ellens, et al. (1984, Biophys. J.. . . hemisuccinate. Brockerhoff and Ramsammy (1982, Biochim. Biophys. Acta. 691, 227) reported that bilayers can be constructed which consist entirely of **cholesterol**, provided that a stabilized hydrophilic anchor is provided. Multilamellar and **unilamellar cholesterol liposomes** have been prepared in a conventional manner. More recently, Janoff et al., U.S. Pat. No. 4,721,612, relevant portions of which. . . Janoff may entrap numerous bioactive agents including insulin, growth hormone, diazepam, indomethacin and tylosin, among others; however, most of the **liposomes** disclosed therein are not storage stable, i.e., capable of remaining

stable for a period of at least one year under. . .

SUMM The present invention relates to a number of **liposome** and **liposome**-like structures (vesicles) formed from derivatized sterols of the present invention. The derivatized sterols according to the present invention have the. . .

SUMM The derivatized sterols of the present invention form **liposomes** and **liposome**-like structures (vesicles) which may function as adjuvants or as delivery vehicles for therapeutic and diagnostic purposes. As used throughout the specification and claims, the term "vesicle" is used to embrace all **liposome** and **liposome**-like structures which may be made with the derivatized sterols of the present invention. The vesicles of the present invention may. . .

SUMM The vesicles may be used to entrap numerous bioactive agents, including immunogens and adjuvants. One particularly advantageous application of the **liposomes** of the present invention includes entrapping insoluble bioactive agents or agents that are only sparingly soluble in water. This advantageously. . .

SUMM 7). Stability is enhanced in comparison to **cholesterol** hemisuccinate (CHS) vesicles.

SUMM . . . of influenza is in combination with the vesicles of the present invention. In another embodiment the dosage form is a **liposome** such as a multilamellar vesicle, particularly those multilamellar vesicles at least about 1 micron in diameter. In certain embodiments the immunogen is entrapped in the **liposome**.

SUMM . . . embodiments of the immunogen dosage form of the present invention, a given dose of the immunogen is entrapped in the **liposome**, preferably a multilamellar vesicle containing an adjuvant amount of the derivatized sterols according to the present invention. Preferably these vesicles. . . combination with an adjuvant amount of a derivatized sterol according to the present invention. In certain cases, small amounts of **cholesterol** may be added as well. Preferred vesicles comprise DMPC/derivatized sterol in a molar ratio of from about 80:20 to about 20:80. Most preferably, the ratio of DMPC/derivatized **cholesterol** is about 40:60 to about 60:40 and the vesicle is a multilamellar vesicle such as an SPLV.

SUMM The present invention relates to novel **liposomes** and **liposome**-like structures (vesicles) comprising an amount of a derivatized sterol either alone or in combination with additional **liposome**-forming lipids.

SUMM . . . fractions such as hemagglutinin, parainfluenza 3 (fusion and hemagglutinin-neuraminidase), malaria sporozoite fractions, hepatitis (A, B, and non-A/non-B) fractions, meningococcus fractions, **HIV** fractions (all strains), and melanoma fractions.

SUMM . . . Preferred multilamellar vesicles are at least about 1 micron in diameter. In certain embodiments the immunogen is entrapped in the **liposome** and in other embodiments the immunogen is ionically associated with the surface of the **liposome** containing the derivatized sterol.

SUMM . . . a human, comprising the step of administering to the animal a priming immunization dose of a composition comprising an a **liposome** adjuvant--any type of **liposome**--and particularly a **liposome** which is a derivatized sterol of the present invention and an adjuvant-obligatory immunogen such that administration of a booster dose. . .

SUMM . . . composition further comprises a vesicle (including multilamellar vesicles) preferably wherein the immunogen is entrapped in the vesicles most preferably in **liposomes** at least about 1 micron in diameter. In employing this method the composition further can comprise a derivitized sterol wherein. . .

SUMM Further, this invention includes a dosage form comprising an immunogen and a multilamellar **liposome** comprising DMPC in an immunization dose in combination with a derivatized sterol according to the present invention. In this aspect. . . produces an adjuvant effect. In further embodiments aluminum adjuvants such as aluminum hydroxide gel are included. In one embodiment the **liposome** of the dosage form comprises DMPC/**cholesterol** in a molar ratio of about 80:20 to about 20:80, preferably about 70:30. In specific embodiments the dosage form multilamellar **liposome** is an SPLV and/or at least 1 micron in diameter

and particularly a SPLV containing SAPC:**cholesterol** in a ratio of about 70:30.

SUMM . . . comprising the step of administering to such animal an immunization dose of a composition comprising an immunogen and a multilamellar **liposome** comprising DMPC in combination with a derivitized sterol of the present invention, and optionally further including aluminum adjuvants such as. . .

SUMM . . . an immune response in an animal including a human comprising the use of an adjuvant wherein the adjuvant comprises a **liposome** comprising DMPC in combination with an adjuvant inducing amount of a derivatized sterol of the present invention, one embodiment further including aluminum adjuvants such as aluminum hydroxide gel. In a preferred embodiment the method utilizes **liposomes** comprising a molar ratio of DMPC/derivatized sterol of about 80:20 to 20:80, most preferably wherein the ratio is from about 30:70 to about 70:30. In specific embodiments of the method the multilamellar **liposome** is an SPLV and/or at least about 1 micron in diameter and particularly a 70:30 DMPC/derivatized sterol SPLV.

SUMM . . . step of administering to the animal a priming immunization dose of a composition comprising an adjuvant which is a multilamellar **liposome** comprising DMPC in combination with an adjuvant inducing amount of a derivatized sterol of the present invention and an adjuvant-obligatory. . .

SUMM . . . fractions such as hemagglutinin, parainfluenza 3 (fusion and hemagglutinin-neuraminidase), malaria sporozoite fractions, hepatitis (A, B, and non-A/non-B) fraction, meningococcus fractions, **HIV** fractions (all strains), and melanoma fractions.

SUMM . . . at least one element of which is administering an immunization dose of a composition comprising an immunogen and a multilamellar **liposome** comprising DMPC in combination with a derivatized sterol of the present invention. In one aspect of the method the composition further comprises aluminum adjuvant such as aluminum hydroxide gel. In this method a particular **liposome** comprises in combination with a derivatized sterol of the present invention in an adjuvant inducing amount DMPC:derivatized sterol in a. . . of about 80:20 to about 20:80, preferably about 70:30 to about 30:70 and most preferably about 70:30 particularly wherein the **liposome** is an SPLV and including the **liposome** being at least about 1 micron in diameter.

SUMM In a further aspect this invention includes a dosage form comprising an immunogen and a **liposome** comprising DMPC in an immunization dose in combination with an adjuvant inducing amount of a derivatized sterol of the present. . .

DETD . . . related to novel vesicles comprising an amount of a derivativized sterol either alone or in combination with at least one **liposome**-forming lipid. The derivatized sterols according to the present invention have the general structures: ##STR2## where A is a core molecule. . .

DETD The derivatized sterols of the present invention form **liposomes** and **liposome**-like structures (vesicles) which may function as adjuvants or as delivery vehicles for therapeutic and diagnostic purposes. As used throughout the specification, the term "vesicle" refers to **liposome** bi-layer structures as well as **liposome**-like structures such as hexagonal phases, micelles and reverse micelles which may be formed by derivatized sterols of the present invention.. . . as more fully described herein. In certain cases where the derivatized sterols of the present invention are used to form **liposomes**, the derivatized sterols do not readily form the **liposomes**. In certain cases lipids which normally do not readily form **liposomes** may be added to the derivatized sterol to induce **liposome** formation. This is an unexpected result.

DETD . . . capable of derivatization to produce the derivatized sterols of the present invention. Such sterols and steroids in general may include **cholesterol**, Vitamin D, phytosterols, (including but not limited to sitosterol, campesterol, stigmasterol and the like). Exemplary steroids and sterols include aldosterone, androsterone, testosterone, estrogen, ergocalciferol, ergosterol, estradiol-17alpha, estradiol-17beta, **cholesterol**, cholic acid, corticosterone, estriol, lanosterol,

lithocholic acid, progesterone, cholesterol, corticol, cortisone, cortisone acetate, cortisol acetate, deoxycorticosterone and estrone, among numerous others, with **cholesterol** being preferred. Exemplary bile acids include taurocholic acid, desoxycholic acid, and geicocholic acid, among others. The core molecule is chosen. . .

DETD . . . a group which is both acid and base stable. Chemical link B is advantageously chosen to promote the stability of **liposomes** during long periods of storage, preferably for periods in excess of two years. Preferred B linkages include ethers, amides and. . .

DETD . . . free carboxylic acid form. During vesicle formation, however, the free carboxylic acid form will undergo salt formation in situ during **liposome** formation. Both the free carboxylic acid form on the derivatized sterol which undergoes salt formation to charged group D in the **liposome** and derivatized sterols wherein the salt formation occurs before incorporation into **liposomes** are contemplated by the present invention.

DETD . . . negatively charged. The group E counterion is selected based upon the ability of the group to enhance the formation of **liposomes** of the present invention. Preferred counterions may be positively or negatively charged. Negatively charged counterions include acetates, sulfates, phosphates, arsenates,. . .

DETD . . . core molecule is generally at the reactive 3 position. The linkage of the anchor molecule to the 3 position of **cholesterol** is often advantageous. However, where the molecule does not contain a reactive 3 position, then a choice should be made. . .

DETD . . . they may be formed from an adjuvant inducing amount of a derivatized sterol in combination with at least one other **liposome**-forming lipid. Vesicles may contain any amount of derivatized sterol and preferably will contain at least about 0.1 mole percent.

DETD Any **liposome**-forming lipid may be used in combination with the derivatized sterols of the present invention to form vesicles. **Liposome** forming lipids which can be used in the vesicles of the present invention include synthetic, semisynthetic or natural phospholipids and may include **phosphatidylcholine** (**PC**), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylinositol(PI), sphingomyelin (SPM) and cardiolipin, among others, including hydrogenated phopholipids, either. . . present invention may also include dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). In other embodiments, distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), or hydrogenated soy **phosphatidylcholine** (HSPC) may also be used. Dimyristoylphosphatidylcholine (DMPC) and diarachidonoylphosphatidylcholine (DAPC) may similarly be used. Due to the elevated transition temperatures. . . T_c or temperatures slightly higher, e.g., up to about 5° C. higher than the T_c, in order to make these **liposomes**. In preferred embodiments, egg **phosphatidylcholine** is used.

DETD . . . to produce a vesicle either alone or in combination with a derivatized sterol of the present invention is termed a **liposome** forming lipid. Any of the above-mentioned phospholipids and derivatized sterols may be used in combination with at least one additional steroidal component selected from the group consisting of **cholesterol**, cholestanol, coprostanol or cholestane. In addition, polyethylene glycol derivatives of **cholesterol** (PEG-cholesterols), as well as the organic acid derivatives of sterols, for example **cholesterol** hemisuccinate (CHS) and other derivatives as disclosed in U.S. Pat. No. 4,721,612 may also be used in combination with any. . .

DETD . . . nasal, oral, vaginal, rectal, gastrointestinal, mucosa, etc.). Such topical application may be in the form of creams or ointments. The **liposome** containing bioactive agent may be administered alone our will generally be administered in admixture with a pharmaceutical carrier selected with. . .

DETD . . . the bromelain fragment. The derivatized sterols of the present invention are also useful pharmaceutical adjuvants, particularly in the form of **liposomes**. DMPC/**cholesterol liposomes** in combination with

the derivatized sterols of the present invention (especially multilamellar **liposomes**) are also useful pharmaceutical adjuvants alone or in combination with aluminum hydroxide gels.

DETD . . . the vesicles will have a net charge or be neutral. Charged vesicles are believed to display superior adjuvancy to neutral **liposomes**.

DETD . . . is preformed by such methods as vortexing, sonication or other methods well known in the art. If desired the resulting **liposomes** may be filtered or sized such as by passing through a filter stack such as a 0.4 or 0.2 um. . . filter (Nuclepore, Pleasanton, CA). Typically better adjuvant response is observed with greater amounts of lipid. Immunogens which partition into the **liposome** lamellae such as melanoma antigen may yield an insufficient immunogenic response without repeated inoculations and additional immuno stimulator. Without being. . . particular theory it is believed that this partitioning results in the limitation of exposure of epitopes externally to the adjuvant **liposomes**. Immunogens may be modified by a number of methods well known in the art such as by amino acid addition. . .

DETD In certain cases, the derivatized sterols of the present invention do not form **liposomes**. In certain of these cases an additional **liposome** forming lipid may be added to the steroidal component to promote the formation of **liposomes**.

DETD . . . combination with an adjuvant inducing amount of a derivatized sterol of the present invention. DMPC/derivatized sterol forms the required multilamellar **liposomes** over a wide range of proportions from about 100:1 (molar) to about 20:80. More preferred is about 70:30 to about. . . mole percent. Additionally other lipids may be admixed with DMPC/derivatized sterol, such as dimyristoyl phosphatidylglycerol, dicetyl phosphate, phosphatidic acid, phosphatidylethanolamine, **phosphatidylcholine** and **cholesterol** hemisuccinate ("CHS"), such as those with sodium ("CHSsodium") or tris(hydroxymethyl) aminomethane ("CHStris") as the counter ion.

DETD DMPC/derivatized sterol multilamellar **liposomes** of the SPLV process are preferred but any other type of **liposome** may be used. The SPLV process generally involves rotoevaporation of lipids in solvent in a round bottom flask to form. . .

DETD The vesicles are conveniently administered in aqueous material. The volume of aqueous material will vary with the particular **liposome** to be administered and is not critical. Generally about 0.5 ml is a convenient **liposome** dosage volume. Typically better adjuvant response is observed with greater amounts of lipid.

DETD Suitable aqueous material for either sterol or DMPC/derivatized sterol containing **liposomes** is saline solution, bovine serum albumen or other well known aqueous pharmaceutical diluents.

DETD The alkoxide of **cholesterol** is produced by the reaction of sodium with **cholesterol** (molar ratio 1:1 in THF). After formulation, the alkoxide is reacted with 4-bromobutyrate, producing the ethyl ester of 4-butanoate by. . . A second possible reagent for the ether synthesis is the epoxide glycidyltrimethylammonium chloride, which when reacted with the alkoxide of **cholesterol** produces the positively charged cholesteryl-2-hydroxy-3-trimethylammoniumpropyl ether. Upon heating the olefin would be produced.

DETD . . . position 3 of the core molecule (A) is formed by nucleophilic substition with a mercaptide. The 3 hydroxy position of **cholesterol** is tosylated (tosyl chloride/pyridine). The 3-0-tosyl group is then reacted with with the salt of a mercaptan, for example, 4-mercaptobutanoic. . .

DETD Grignard reagents could also be employed to provide the thio ethers. Reaction of **cholesterol** disulfide with magnesium 2(2-bromoethyl)-1,3-dioxolane yields the sulfide of **cholesterol** and the dioxolane. Mild acid hydrolysis and mild oxidation (oxygen) of the resulting aldehyde would give the carboxylic acid cholesteryl. . .

DETD Alkylation of **cholesterol** and other core molecules (A) of the present invention may be performed in a wide variety of ways. A number of positions other than the 3 position of **cholesterol** may be alkylated readily.

DETD . . . quaternium derivative of cholesteryl and an epoxide such as glycidotrimethyl ammonium chloride to produce the positively charged alkyl derivative of **cholesterol** B-hydroxy propyltrimethylammonium.

DETD Preparation of Adjuvant **Liposomes** with Antigen

DETD . . . intermittently vortexed over a 2-hour period at 22.5° C.+2.5° C. and left until no large clumps are visible. The resultant **liposomes** are washed 3 times in 10 ml of aqueous buffer solution being separated each time by 15 minutes of centrifugation. . .

DETD Preparation of Adjuvant **Liposomes** with Antigen

DETD Preparation of Adjuvant **Liposomes** with Antigen

DETD Preparation of Adjuvant **Liposomes** with Antigen

DETD Preparation of Gel Admixed with **Liposomes**

DETD . . . 2% (Alhydrogel.TM.; Connaught Laboratories, Inc., Swiftwater, PA) containing 7.29 mg/ml aluminum is used in conjunction with 1.02 ml of the **liposomes** of this invention prepared as in Example 9. The **liposomes** are admixed with 0.67 ml aluminum hydroxide gel and 5.31 ml of saline. The final aluminum concentration is 0.7 mg/ml. . .

DETD . . . 0.01 M TRIS-HCl (pH approximately 7.3). The resulting pellet, which is red in color, indicates entrapment of the arsenazo III. **Liposomes** prepared from other derivatized sterols wherein the salt may be prepared as described above, or by following the procedure of. . .

DETD . . . obvious modification. In addition to numerous bioactive agents, inulin, chromium, growth hormone, insulin, tylosin, and diazepam may be entrapped in **liposomes** prepared from the derivatized sterols of the present invention. Therapeutic methodology in utilizing bioactive agents entrapped in **liposomes** closely adheres to the teachings of the art.

CLM What is claimed is:

6. The vesicles according to claim 1 wherein the vesicles are **unilamellar** vesicles.

. . . wherein said core molecule (A) is selected from the group consisting of aldosterone, androsterone, testosterone, estrogen, ergocalciferol, ergosterol, estradiol-17alpha, estradiol-17beta, **cholesterol**, cholic acid, corticosterone, estriol, lanosterol, lithocholic acid, progesterone, cholecalciferol, cortisol, cortisone, cortisone acetate, cortisol acetate, deoxycorticosterone, estrone, and phytosterols.

9. The vesicles according to claim 8 wherein said core molecule (A) is **cholesterol**.

14. The method according to claim 10 wherein said vesicles are **unilamellar** vesicles.

. . . wherein said core molecule (A) is selected from the group consisting of aldosterone, androsterone, testosterone, estrogen, ergocalciferol, ergosterol, estradiol-17alpha, estradiol-17beta, **cholesterol**, cholic acid, corticosterone, estriol, lanosterol, lithocholic acid, progesterone, cholecalciferol, cortisol, cortisone, cortisone acetate, cortisol acetate, deoxycorticosterone, estrone, and phytosterols.

17. The method according to claim 16 wherein said core molecule (A) is **cholesterol**.

22. The dosage form according to claim 21 wherein said antigen is selected from the group consisting of influenza virus. . . fractions, parainfluenza 3, malaria sporozoite fractions, hepatitus A virus fractions, hepatitis B virus fractions, hepatitis non-A/non-B virus fractions, meningococcus fractions, **HIV** fractions and melanoma cell fractions.

. . . according to claim 18 wherein said vesicle comprises an adjuvant inducing amount of said derivatized sterol in combination with a **liposome** forming lipid.

29. The dosage form according to claim 28 wherein said **liposome**

forming rapid 1,2-dimyristoylphosphatidylcholine.

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=> d his

     (FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004)

     FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004
L1         33450 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L2          3069 S L1 AND (ALUMINUM HYDROXIDE)
L3          1785 S L1 AND (UNILAMELLAR)
L4          1772 S L3 AND LIPOSOME?
L5           434 S L4 AND (PHOSPHATIDYLCHOLINE)
L6           402 S L5 AND CHOLESTEROL
L7           107 S L6 AND MUCOSAL
L8            83 S L7 AND ORAL
L9            22 S L8 AND AY<2000
L10        13577 S L1 AND LIPOSOME?
L11          897 S L10 AND LIPOSOME?/CLM
L12          244 S L11 AND UNILAMELLAR
L13           18 S L12 AND UNILAMELLAR/CLM
L14           17 S L13 AND (CHOLESTEROL)
L15           17 S L14 AND (PHOSPHATIDYLCHOLINE OR PHOSPHATIDYL CHOLINE OR PC)
L16            2 S L15 AND IMMUNOGENIC/CLM
L17           15 S L15 NOT L16

=> e friede martin/in
E1             1     FRIEDE KLAUS/IN
E2             1     FRIEDE MANFRED/IN
E3            11 --> FRIEDE MARTIN/IN
E4             6     FRIEDE MATTHEW D/IN
E5             3     FRIEDE PATRICK W/IN
E6             1     FRIEDE PETRA/IN
E7             2     FRIEDE STEVE G/IN
E8             1     FRIEDE WOLFGANG/IN
E9             1     FRIEDEBACH A HANS/IN
E10           11     FRIEDEBACH ADOLF H/IN
E11            1     FRIEDEBACH ADOLPH H/IN
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=> s e3
L18           11 "FRIEDE MARTIN"/IN

=> s l18 and ay<2000
        2994935 AY<2000
L19            3 L18 AND AY<2000

=> d l19,cbib,ab,clm,1-3

L19  ANSWER 1 OF 3  USPATFULL on STN
2003:123086 Vaccine adjuvants.
    Friede, Martin, Court St Etienne, BELGIUM
    Hermand, Philippe, Court St Etienne, BELGIUM
    SmithKline Beechman Biologicals s.a., Rixensart, BELGIUM (non-U.S.
    corporation)
    US 6558670 B1 20030506
    APPLICATION: US 1999-301829  19990429 (9)                    <--
    PRIORITY: BE 1999-8885  19990419
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB     The present invention relates to adjuvant compositions which are
       suitable to be used in vaccines. In particular, the adjuvant
       compositions of the present invention comprises a saponin and an
       immunostimulatory oligonucleotide, preferably the saponins used in said
       adjuvant combinations are haemolytic. Also provided by the present
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invention are vaccines comprising the adjuvants of the present invention and an antigen. Further provided are methods of manufacture of the adjuvants and vaccines of the present invention and their use as medicaments.

CLM What is claimed is:

1. An immunogenic composition comprising a saponin an immunostimulatory oligonucleotide comprising an unmethylated CG dinucleotide and a tumor-associated antigen.

2. An immunogenic composition as claimed in claim 1 wherein said saponin is QS21.

3. An immunogenic composition as claimed in claim 1 wherein said immunostimulatory oligonucleotide comprises a sequence of XXCGYY, wherein X is a purine and Y is a pyrimidine.

4. An immunogenic composition as claimed in claim 1 wherein said immunostimulatory oligonucleotide is selected from the group comprising: TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1); TCT CCC AGC GTG CGC CAT (SEQ ID NO:2); ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO:3).

5. A method of treatment of an individual susceptible to or suffering from a disease by the administration to an individual an immunogenic composition as claimed in any of claims 1 to 4.

L19 ANSWER 2 OF 3 USPATFULL on STN

2003:13075 Vaccine comprising an iscom consisting of sterol and saponin which is free of additional detergent.

Friede, Martin, Farnham, UNITED KINGDOM

Garcon, Nathalie, Wavre, BELGIUM

SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S. corporation)

US 6506386 B1 20030114

WO 2000007621 20000217

APPLICATION: US 2001-744800 20010604 (9) <--

WO 1999-EP5587 19990803

PRIORITY: GB 1998-17052 19980805

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention provides an improved adjuvant formulation and a process for producing said adjuvant. The adjuvant comprises an ISCOM structure comprising a saponin, said ISCOM structure being devoid of additional detergent.

CLM What is claimed is:

1. An adjuvant composition comprising a sterol, a saponin, and a phospholipid, characterised in that the adjuvant is in the form of an ISCOM and that it is free of additional detergent, other than the saponin.

2. An adjuvant composition as claimed in claim 1, wherein the ratio of saponin:sterol (w/w) exceeds 1.

3. An adjuvant composition as claimed in claim 1, wherein the ratio of saponin to sterol is in the range of 1:1 to 100:1 (w/w).

4. An adjuvant composition as claimed in claim 1, wherein the ratio of saponin to sterol is 5:1.

5. An adjuvant composition as claimed in any one of claims 1 to 4, wherein the saponin is Quil A or extract thereof.

6. An adjuvant composition as claimed in claim 5, wherein the extract of Quil A is QS21.

7. An-adjuvant composition as claimed in claim 1, wherein the sterol is

cholesterol.

8. An adjuvant composition as claimed in claim 1, wherein the phospholipid is phosphatidylcholine.

9. An adjuvant composition as claimed in claim 8, wherein phosphatidylcholine is dioloeoylphosphatidylcholine or dilauryl phosphatidylcholine.

10. An adjuvant composition as claimed in claim 7, wherein the ratio of cholesterol to phospholipid is 50% (w/w).

11. An adjuvant composition as claimed in claim 10, wherein the ratio of cholesterol to phospholipid is 20-25% (w/w).

12. A vaccine comprising an adjuvant composition as claimed in any one of claims 1 to 11, further comprising an antigen.

13. A vaccine composition as claimed in claim 12, wherein the antigen is an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C, or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium, or Toxoplasma.

14. A process for the manufacture of an adjuvant composition, comprising the following steps: (a) the formation of cholesterol containing small unilamellar liposomes (SUL) in the absence of detergent; and (b) admixing the preformed liposomes with saponin at a ratio of saponin:cholesterol (w/w) exceeding 1.

15. A process for the manufacture of a vaccine composition, comprising the following steps: (a) taking an adjuvant composition produced according to the process of claim 14; and (b) adding an antigen or an antigenic composition.

L19 ANSWER 3 OF 3 USPATFULL on STN
2002:346653 Method to enhance an immune response of nucleic acid vaccination.
Dalemans, Wilfried, Hoegaarden, BELGIUM
Van Mechelen, Marcelle, Wagnelee, BELGIUM
Bruck, Claudine, Rixensart, BELGIUM
Friede, Martin, Farnham, UNITED KINGDOM
SmithKline Beecham Biologicals, S.A., Rixensart, BELGIUM (non-U.S. corporation)
US 6500432 B1 20021231
WO 9930733 19990624
APPLICATION: US 2000-581368 20000612 (9) <--
WO 1998-EP8152 19981211
PRIORITY: GB 1997-26555 19971216
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB This invention provides a method to enhance an immune response of nucleic acid vaccination by simultaneous administration of a polynucleotide and a polypeptide of interest.
CLM What is claimed is:
1. A method for enhancing an immune response in an subject wherein the immune response is induced by administration of a polynucleotide, the method comprising the steps of: (1) administering a polynucleotide encoding a polypeptide; and (2) administering the polypeptide encoded by the polynucleotide administered in (1) wherein the polypeptide is administered 1-10 days after the polynucleotide is administered and wherein the method results in an increase in the immune response to the polypeptide as measured by one or more members selected from the group

(iii) cyctotoxic T cell level when compared to the immune response induced by administration of the polynucleotide in the absence of administration of the polypeptide.

2. The method of claim 1 wherein the polypeptide is administered 3-7 days after the polynucleotide is administered.

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=> d his

     (FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004)

     FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004
L1         33450 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L2          3069 S L1 AND (ALUMINUM HYDROXIDE)
L3          1785 S L1 AND (UNILAMELLAR)
L4          1772 S L3 AND LIPOSOME?
L5           434 S L4 AND (PHOSPHATIDYLCHOLINE)
L6           402 S L5 AND CHOLESTEROL
L7           107 S L6 AND MUCOSAL
L8            83 S L7 AND ORAL
L9            22 S L8 AND AY<2000
L10        13577 S L1 AND LIPOSOME?
L11          897 S L10 AND LIPOSOME?/CLM
L12          244 S L11 AND UNILAMELLAR
L13           18 S L12 AND UNILAMELLAR/CLM
L14           17 S L13 AND (CHOLESTEROL)
L15           17 S L14 AND (PHOSPHATIDYLCHOLINE OR PHOSPHATIDYL CHOLINE OR PC)
L16            2 S L15 AND IMMUNOGENIC/CLM
L17           15 S L15 NOT L16
                 E FRIEDE MARTIN/IN
L18           11 S E3
L19            3 S L18 AND AY<2000

=> s (vaccin? or immunogenic composition?)
          37291 VACCIN?
          26644 IMMUNOGENIC
         799547 COMPOSITION?
           2274 IMMUNOGENIC COMPOSITION?
                  (IMMUNOGENIC(W)COMPOSITION?)
L20       37491 (VACCIN? OR IMMUNOGENIC COMPOSITION?)

=> s l20 and liposome?
          42761 LIPOSOME?
L21       17084 L20 AND LIPOSOME?

=> s l21 and unilamellar
           5574 UNILAMELLAR
L22        1926 L21 AND UNILAMELLAR

=> s l22 and (phosphatidylcholine or phosphatidyl choline or PC)
           8350 PHOSPHATIDYLCHOLINE
           6952 PHOSPHATIDYL
          18412 CHOLINE
           3334 PHOSPHATIDYL CHOLINE
                  (PHOSPHATIDYL(W)CHOLINE)
         116449 PC
L23        1153 L22 AND (PHOSPHATIDYLCHOLINE OR PHOSPHATIDYL CHOLINE OR PC)

=> s l23 and cholesterol
          36219 CHOLESTEROL
L24        1063 L23 AND CHOLESTEROL

=> s l24 and (70 nm or 150 nm)
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        284026 NM
          6405 70 NM
                 (70(W)NM)
        920033 150
        284026 NM
         12768 150 NM
                 (150(W)NM)
L25         53 L24 AND (70 NM OR 150 NM)

=> s l25 and (HIV or human immunodeficiency virus)
         31752 HIV
        394447 HUMAN
         18315 IMMUNODEFICIENCY
         75322 VIRUS
         13121 HUMAN IMMUNODEFICIENCY VIRUS
                 (HUMAN(W)IMMUNODEFICIENCY(W)VIRUS)
L26         34 L25 AND (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)

=> s l26 and ay<2000
       2994935 AY<2000
L27          8 L26 AND AY<2000

=> d l27,cbib,ab,clm,kwic,1-8

L27  ANSWER 1 OF 8  USPATFULL on STN
2001:93121 Reconstitution of purified membrane proteins into preformed
    liposomes.
    Singh, Pratap, Wilmington, DE, United States
    Dade Behring Inc., Deerfield, IL, United States (U.S. corporation)
    US 6248353 B1 20010619
    APPLICATION: US 1999-459137  19991210 (9)                    <--
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB     The present application relates to a method of making liposomes having
       membrane proteins incorporated therein, the method comprising: providing
       the membrane protein in solution; providing a solution of preformed
       liposomes; and incubating the mixture. Prior to the step of providing
       a solution of preformed liposomes, the liposomes are formed by
       combining a mixture of phospholipids with a solution of at least one
       type of unsaturated fatty acid. The methods of the present invention
       further relate to the method of making a reagent comprising tissue
       factor reconstituted into preformed liposomes. The method of the
       present invention for making a tissue factor reagent comprises:
       providing tissue factor in solution; providing a solution of preformed
       liposomes comprising a mixture of phospholipids and at least one type
       of unsaturated fatty acid; and incubating the mixture.
CLM    What is claimed is:
       1. A method of making liposomes having membrane proteins incorporated
       therein, the method comprising: a) providing the membrane protein in
       solution; b) providing a solution of preformed liposomes wherein the
       liposomes are formed by combining a mixture of phospholipids with a
       solution of at least one type of unsaturated fatty acid; and c)
       incubating the membrane protein solution of a) with the solution of
       preformed liposomes of b).

       2. The method according to claim 1, wherein the fatty acid comprises an
       unsaturated fatty acid having from about 16 to about 20 carbon atoms.

       3. The method according to claim 2, wherein the fatty acid comprises
       oleic acid.

       4. The method according to claim 1, wherein the step of providing the
       membrane protein in solution further comprises solubilizing the membrane
       protein in a salt solution.
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5. The method according to claim 1, wherein the mixture of phospholipids comprises dioleoylphosphatidylcholine and dioleoylphosphatidylserine.

6. The method according to claim 5, wherein the dioleoylphosphatidylcholine and dioleoylphosphatidylserine are in a ratio of from about 4 to about 1.

7. The method according to claim 6, wherein the dioleoylphosphatidylcholine and dioleoylphosphatidylserine are in a ratio of from about 7 to about 3.

8. The method according to 1, wherein the phospholipids are synthetic.

9. The method according to claim 1, wherein the solutions are incubated at about 25° C. to about 45° C.

10. The method according to claim 3, wherein the oleic acid is present in an amount ranging from about 15 to about 30 weight percent.

11. The method according to claim 10, wherein the oleic acid is present in an amount about 16 weight percent.

12. A method of making a tissue factor reagent comprising: a) providing tissue factor in solution; b) providing a solution of preformed **liposomes** wherein the **liposomes** are formed by combining a mixture of phospholipids with a solution of at least one type of unsaturated fatty acid; and c) incubating the membrane protein solution of a) with the solution of preformed **liposomes** of b).

13. The method according to claim 12, wherein the fatty acid comprises an unsaturated fatty acid having from about 16 to about 20 carbon atoms.

14. The method according to claim 13, wherein the fatty acid comprises oleic acid.

15. The method according to claim 12, wherein the step of providing the membrane protein in solution further comprises solubilizing the membrane protein in a salt solution.

16. The method according to claim 12, wherein the mixture of phospholipids comprises dioleoylphosphatidylcholine and dioleoylphosphatidylserine.

17. The method according to claim 16, wherein the dioleoylphosphatidylcholine and dioleoylphosphatidylserine are in a ratio of from about 4 to about 1.

18. The method according to claim 17, wherein the dioleoylphosphatidylcholine and dioleoylphosphatidylserine are in a ratio of from about 7 to about 3.

19. The method according to 12, wherein the phospholipids are synthetic.

20. The method according to claim 12, wherein the solutions are incubated at about 25° C. to about 45° C.

21. The method according to claim 14, wherein the oleic acid is present in an amount ranging from about 15 to about 30 weight percent.

22. The method according to claim 21, wherein the oleic acid is present in an amount about 16 weight percent.

TI Reconstitution of purified membrane proteins into preformed **liposomes**

AI US 1999-459137 19991210 (9) <--

AB The present application relates to a method of making **liposomes** having membrane proteins incorporated therein, the method comprising: providing

the membrane protein in solution; providing a solution of preformed **liposomes**; and incubating the mixture. Prior to the step of providing a solution of preformed **liposomes**, the **liposomes** are formed by combining a mixture of phospholipids with a solution of at least one type of unsaturated fatty acid.. . . methods of the present invention further relate to the method of making a reagent comprising tissue factor reconstituted into preformed **liposomes**. The method of the present invention for making a tissue factor reagent comprises: providing tissue factor in solution; providing a solution of preformed **liposomes** comprising a mixture of phospholipids and at least one type of unsaturated fatty acid; and incubating the mixture.

SUMM The present invention relates to methods of incorporating membrane proteins into preformed **liposomes**. It further relates to methods of making a prothrombin time (PT) reagent using purified, reconstituted natural or recombinant human tissue factor (rTF). More particularly, the invention relates to the reconstitution of tissue factor (TF) into phospholipid **liposomes** to produce a tissue factor-based PT reagent.

SUMM **Liposomes** are a general category of vesicles which comprise one or more lipid bilayers surrounding an aqueous space. **Liposomes** include **unilamellar** vesicles composed of a single membrane or lipid bilayer, and multilamellar vesicles (MLVs) composed of many concentric membranes (or lipid bilayers). **Liposomes** are commonly prepared from phospholipids. Due to unique characteristics of these vesicles, **liposomes** have been widely used as a model membrane for investigating the properties of biomembranes and for studying the functions of. . .

SUMM There are essentially four presently known mechanisms for incorporating, i.e., reconstituting, proteins into **liposomes**. See Rigaud, J- L., et al., "**Liposomes** as Tools for the Reconstitution of Biological Systems," p. 71-88, in **Liposomes** as Tools in Basic Research and Industry, ed. Philippot, J. R. and Schuber, F., CRC Press, Boca Raton, Fla. (1995).. . . procedures often result in the denaturation of the proteins. A second method uses mechanical means to produce large and small **unilamellar** vesicles from MLVs by swelling of the dry phospholipid films in excess buffer. Such mechanical means include sonication of MLVs,. . . or dehydration-rehydration. Drawbacks with sonication include variability and inactivation of certain proteins by sonication as well as production of small **liposomes**. A third process involves the direct incorporation of proteins into preformed small **unilamellar liposomes**, also termed spontaneous incorporation. Such methods are usually catalyzed by low cholate or lysolecithin concentrations. Problems with these methods include the wide size distribution of the proteoliposomes, heterogeneous distribution of the protein among the **liposomes** and presence of the non-phospholipid impurities, required for an effective protein incorporation, that would affect performance of those **liposomes**. The fourth and most often used method of incorporating proteins into **liposomes** involves the use of detergents. In such a method, the proteins and phospholipids are cosolubilized in a detergent to form. . . then removed, resulting in the spontaneous formation of bilayer vesicles with the protein incorporated therein. The detergent is incorporated into **liposome** as well as the protein and thus, these methods require removal of the detergent by methods such as dialysis, gel. . . is also difficult to remove completely. Another disadvantage is that one cannot control the orientation of protein incorporated into the **liposomes** by using the detergent methods.

SUMM **Liposomes** have several properties which make them useful in various applications. The most important of these characteristics are the uniform controllable size and the surface characteristics which can control the biological fate of the **liposomes**. These properties make **liposomes** preferred carriers for drug delivery systems and the basis for reagents for assays. For example, **liposomes** containing tissue factor have been used as reagents for prothrombin time (PT) assays for testing coagulation of blood. In these cases, the phospholipid constituent of the **liposomes** is used as a substitute for platelet phospholipids, which are essential for normal hemostasis in vivo. For example, Dade Behring. . .

SUMM . . . reliquidization, the membrane protein is incorporated into a lipid membrane in active form. Suitable additives can be added and the **liposomes** subjected to further processing. If tissue factor apoprotein is relipidized using one of the processes according to the invention, its. . .

SUMM As mentioned above, INNOVIN® is one example of a commercial product in which a membrane protein is incorporated into **liposomes**. Because INNOVIN® is manufactured from recombinant human tissue factor and synthetic phospholipid, it does not contain any other clotting factors,. . . seasonal variability, lot-to-lot variability and is dependent on reliable raw material sources. Human tissue factor may be a source of **HIV** or other human viral diseases and is also dependent on reliable sources. Ox brain gives normal PT values that are. . .

SUMM The present invention relates to methods for reconstituting purified membrane proteins into preformed **liposomes**, in the presence of at least one type of fatty acid. In one preferred embodiment, the present invention relates to. . .

SUMM The present application relates to a method of making **liposomes** having membrane proteins incorporated therein, the method comprising: providing the membrane protein in solution; providing a solution of preformed **liposomes**; and incubating the mixture under physiological conditions of temperature and pH. Prior to the step of providing a solution of preformed **liposomes**, the **liposomes** are formed by combining a mixture of phospholipids with a solution of at least one type of unsaturated fatty acid.. . .

SUMM The preformed **liposomes** for use in the methods of the present invention comprise either natural or synthetic phospholipids. The mixture of phospholipids comprises. . . dilinoleoylphosphatidylserine , dilinoleoylphosphatidylethanolamine, dilinoleoylphosphatidylglycerol, dilinoleoylphosphatidic acid, palmiticlinoleoylphosphatidylcholine, palmiticlinoleoylphosphatidylserine, palmiticlinoleoylphosphatidylethanolamine, palmiticlinoleoylphosphatidylglycerol, palmiticlinoleoylphosphatidic acid. These phospholipids may also be the monoacylated derivatives of **phosphatidylcholine** (lysophophatidylidylcholine), phosphatidylserine (lysophosphatidylserine), phosphatidylethanolamine (lysophosphatidylethanolamine), phophatidylglycerol (lysophosphatidylglycerol) and phosphatidic acid (lysophosphatidic acid). The monoacyl chain in these lysophosphatidyl derivatives may. . .

SUMM As mentioned above, the method of the present invention involves the step of incubating the mixture of membrane protein and **liposomes** between 25° and 45° C. but preferably at about 37° C. Preferably the mixture is heated from about 30 minutes. . .

SUMM . . . methods of the present invention further relate to the method of making a reagent comprising tissue factor reconstituted into preformed **liposomes**. The method of the present invention for making a tissue factor reagent comprises: providing tissue factor in solution; providing a solution of preformed **liposomes** comprising a mixture of phospholipids and at least one type of unsaturated fatty acid; and incubating the mixture under the. . .

SUMM The methods of the present invention provide a method to combine membrane proteins, e.g., tissue factor, with preformed **liposomes** which is more efficient and reproducible than presently used methods. The present invention also relates to a PT reagent which. . .

SUMM The present invention relates to methods of reconstituting purified membrane proteins into preformed **liposomes**. The present methods enable such reconstitution without the use of detergent, as required in presently heretofore known methods. The term. . .

SUMM In one embodiment of the present invention, the protein that is incorporated into the **liposomes** is a purified tissue factor. The protein is active as demonstrated by showing clotting activity which is comparable to that of tissue factor in **liposomes** obtained by previously known methods, e.g., by using detergent.

SUMM . . . 5,599,909). However, the disadvantage of this method is that low pH heating may cause aggregation and irreversible denaturation of

the **liposomes** and irreversible denaturation of the proteins. Another popular method involves the use and removal of detergent. Disadvantages of this method. . . and phase change that takes place during this process slows detergent removal even more. The detergent may be incorporated into **liposome**, in addition to the protein, which may lead to leaky and fragile **liposomes**. The detergent is difficult to remove completely and finally, one cannot control the orientation of protein incorporated into the **liposomes**.

SUMM The methods of the present invention enable the reconstitution of purified membrane proteins into preformed **liposomes** without the need to use detergent for the step of reconstitution.

SUMM As described briefly above, the present methods of making **liposomes** having membrane proteins incorporated therein, comprise the steps of providing the membrane protein in solution; providing a solution of preformed **liposomes**; and incubating the mixture under physiological conditions. The preformed **liposomes** are made by a method comprising combining a mixture of phospholipids with a solution of at least one type of. . .

SUMM The phospholipids which are used to form the preformed **liposomes** in the methods of the present invention comprise either natural or synthetic phospholipids. The phospholipids are selected from phospholipids containing. . . dilinoleoylphosphatidylserine, dilinoleoylphosphatidylethanolamine, dilinoleoylphosphatidylglycerol, dilinoleoylphosphatidic acid, palmiticlinoleoylphosphatidylcholine, palmiticlinoleoylphosphatidylserine, palmiticlinoleoylphosphatidylethanolamine, palmiticlinoleoylphosphatidylglycerol, palmiticlinoleoylphosphatidic acid These phospholipids may also be the monoacylated derivatives of **phosphatidylcholine** (lysophophatidylidylcholine), phosphatidylserine (lysophosphatidylserine), phosphatidylethanolamine (lysophosphatidylethanolamine), phophatidylglycerol (lysophosphatidylglycerol) and phosphatidic acid (lysophosphatidic acid). The monoacyl chain in these lysophosphatidyl derivatives may. . .

SUMM . . . in the range from about 25 to 35% of total phospholipid with the most preferred at about 30% and natural **phosphatidyl choline** (**PC**) in the range from about 65 to 75% of total phospholipid with the most preferred at about 70%. The **phosphatidylcholine** used is neutral in charge, while the phosphatidylserine is negatively charged. In the preferred embodiment the lipid mixture has at. . . other lipids. A preferred source of the natural PS is from bovine brain and a preferred source of the natural **PC** is from egg yolk.

SUMM . . . unsaturated fatty acid side chains with C14, C16, C18 or C20 chains length in either or both the PS or **PC**. Preferred compositions include but are not limited to those that have dioleoyl (18:1)-PS; palmitoyl (16:0)-oleoyl (18:1)-PS, dimyristoyl (14:0)-PS, dipalmitoleoyl (16:1)-**PC**, dipalmitoyl (16:0)-**PC**, dioleoyl (18:1)-**PC**, palmitoyl (16:0)-oleoyl (18:1)-**PC**, and myristoyl (14:0)-oleoyl (18:1)-**PC** as constituents.

SUMM . . . ratio being about 1:10,000. This leads to a final concentration of about 50-300 M of total phospholipids. Thus both the PS:**PC** and rTF to total phospholipid ratio are essential to achieve and maintain optimal functional activity.

SUMM As aforesaid, the methods of the present invention utilize preformed **liposomes** made from phospholipids and fatty acids. Preferably, the fatty acid comprises an aliphatic unsaturated fatty acid having from about 16. . .

SUMM The present invention can be used to reconstitute other membrane proteins into preformed **liposomes**. The proteins useful in the methods of the present invention have a similar structure and biological properties to those of. . .

SUMM The preferred embodiment of the present invention uses a well-defined, purified proteins, e.g., rTF, in combination with **liposomes** formed from purified, well-defined phospholipids and a fatty acid. Full length as well as truncated recombinant molecules can be used. . .

SUMM In the methods of the present invention, the solubilized membrane

protein is mixed with the preformed **liposomes** and incubated under physiological conditions. The incubation preferably occurs at or near the transition temperature of the **liposomes**, e.g., between 25-37° C. In a preferred embodiment which uses a mixture of dioleoylphosphatidylcholine and dioleoylphosphatidylserine (in a ratio of. . .

SUMM Preferably, the **liposomes** used have a size ranging from 75 to **150 nm**, most preferably about 100 nm. It is preferred that the **liposomes** have a generally uniform size. This uniformity can be obtained by methods known in the art. Table 1 shows the effect of **liposome** size on clotting time. The smaller and larger **liposomes**, e.g., 50 nm and 400 nm, show a longer clotting time. In this table the **liposomes** were made from 3.15 nM protein /75 μM phospholipid mixture and sized by extrusion through appropriate size membrane (Avestin, Inc.,. . .

SUMM TABLE 1

Effect of size of **liposomes** on the clotting time

Size of preformed **liposomes**	Clotting time (seconds)
50 nm	34.3
100 nm	28.7
400 nm	54.4

SUMM . . . present invention further relate to the method making reagents for assays comprising proteins useful for those assays reconstituted into preformed **liposomes**. For example, the reconstituted **liposomes** can be used for receptor dependent or receptor independent drug delivery, e.g., delivery of receptor independent delivery of growth hormones. These **liposomes** can also be used as carriers of antigens for antibody production, gene transfer agents, in **vaccine** manufacture for entrapping viral proteins and to study of enzyme isoforms, including free and membrane bound enzymes with distinctly different physiological functions. The **liposomes** can also be used to study enzyme catalyzed reactions, especially if the product acts as a very potent inhibitor of. . .

SUMM . . . methods of the present invention further relate to the method of making a reagent comprising tissue factor reconstituted into preformed **liposomes**. The method of the present invention for making a tissue factor reagent comprises: providing tissue factor in solution; providing a solution of preformed **liposomes**; and incubating the mixture under physiological conditions. The step of providing a solution of preformed **liposomes** further comprises forming the **liposomes** by combining a mixture of phospholipids with a solution of at least one type of unsaturated fatty acid. The mixture of phospholipids preferably comprises a mixture of phospholipids selected from dioleoylphosphatidylcholine, dioleoylphosphatidylserine, and dioleoylphosphatidylethanolamine. Preferably, the **liposomes** are formed from a mixture of dioleoylphosphatidylcholine and dioleoylphosphatidylserine and oleic acid. Preferably the dioleoylphosphatidylcholine and dioleoylphosphatidylserine are present in a ratio of 7:3 and the oleic acid is present at 16% (w/w). The **liposome** prepared from a solution of the phospholipid/oleic acid solution is combined with a solution of tissue factor, solubilized in salt. . .

SUMM If desired, the resulting solution of reconstituted **liposomes** is then diluted with an appropriate buffer. One example of a useful buffer includes, Hepes-based buffer, pH 7.4, containing salt,. . .

SUMM The methods of the present invention provide a method to combine membrane proteins, e.g., tissue factor, with preformed **liposomes** which is more efficient and reproducible than presently used methods. The present invention also relates to a PT reagent which. . .

SUMM The PT reagents of the present invention are very stable. Once the reconstituted **liposomes** are formed, they are stable in solution especially if the solution is maintained below the transition temperature of the **liposomes**. For example, if the transition temperature of the **liposomes** is about 37° C., then the reconstituted **liposome** solution is preferably maintained at about 4° C. to about 25° C.

DETD I. Reconstitution of Purified Tissue Factor into Preformed **Liposomes**

Without Added Fatty Acid

DETD B. Preparation of **Liposomes**

DETD The final mixture containing **liposomes** reconstituted with tissue factor were tested on MLA ELECTRA 900CTM.

DETD II. Reconstitution of Purified Tissue Factor into Preformed **Liposomes** with Added Fatty Acid

DETD B. Preparation of **Liposomes**

DETD The final mixture containing **liposomes** reconstituted with tissue factor were tested on MLA ELECTRA 900C.TM..

DETD When the final mixture containing **liposomes** reconstituted with tissue factor was tested on MLA ELECTRA 900C.TM., the solution showed clotting times of 12.5 and 39.4 sec., when used with normal frozen plasma and Coumadine plasma, respectively. For this experiment the **liposomes** contained 58, 25 and 17% (w/w) of DOPC, DOPS and oleic acid respectively. Under identical conditions, a control (TFS-625-manufactured lot. . .

DETD . . . buffer was used to exchange a detergent solution of the protein; the clear protein solution was then incubated with the **liposome** emulsion in 40 mM Hepes-160 mM NaCl, pH 7.40.

[f] Clotting time greater than the limit of the instrument.

DETD C. Effect of **Liposome** Additives on the Clotting Time

DETD Table 4 also shows that additives, such as **cholesterol** and palmitic acid increase clotting times.

DETD TABLE 4

Effect of **liposome** additives on the clotting time

Liposome additives	Clotting time (sec)[1]	Clotting time (sec)[2]	Clotting time (sec)[3]
DOPC + DOPS + **Cholesterol** (16%)	23.0		
DOPC + DOPS + Palmitic acids (16%)	29.9		
DOPC + DOPS + Oleic acid (16%)	16.4		14.1
DOPC + DOPS + choles.. . .			

DETD TABLE 5

Liposome additive	Clotting time (sec)
DOPS + (DOPC + DOPE (10%))	28.5
DOPS + (DOPC + DOPE (20%))	24.1
DOPS + (DOPC. . .	

DETD TABLE 5

Liposome additive	Clotting time (sec)
DOPS + (DOPC + DOPE (10%))	28.5
DOPS + (DOPC + DOPE (20%))	24.1
DOPS + (DOPC. . .	

1. A method of making **liposomes** having membrane proteins incorporated therein, the method comprising: a) providing the membrane protein in solution; b) providing a solution of preformed **liposomes** wherein the **liposomes** are formed by combining a mixture of phospholipids with a solution of at least one type of unsaturated fatty acid; and c) incubating the membrane protein solution of a) with the solution of preformed **liposomes** of b).

. . . method of making a tissue factor reagent comprising: a) providing tissue factor in solution; b) providing a solution of preformed **liposomes** wherein the **liposomes** are formed by combining a mixture of phospholipids with a solution of at least one type of unsaturated fatty acid; and c) incubating the membrane protein solution of a) with the solution of preformed **liposomes** of b).

L27 ANSWER 2 OF 8 USPATFULL on STN

2000:174129 Preparation for the application of agents in mini-droplets.

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DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The invention relates to a preparation for the application of agents in the form of minuscule droplets of fluid, in particular provided with membrane-like structures consisting of one or several layers of amphiphilic molecules, or an amphiphilic carrier substance, in particular for transporting the agent into and through natural barriers such as skin and similar materials. The preparation contains a concentration of edge active substances which amounts to up to 99 mol-% of the agent concentration which is required for the induction of droplet solubilization. Such preparations are suitable, for example, for the non-invasive applications of antidiabetics, in particular of insulin. The invention, moreover, relates to the methods for the preparation of such formulations.

CLM What is claimed is:

1. A method of transporting medical agents through the skin of a mammal, comprising (A) preparing transfersomes comprising a pharmaceutically acceptable lipid and a pharmaceutically acceptable surfactant which is compatible with said lipid, said transfersomes being contained in a pharmaceutically acceptable medium for application onto said skin, said transfersomes containing said lipid and said surfactant in a ratio which enables said transfersomes to undergo sufficient deformation to enable said transfersomes to pass through the skin of said mammal as an entity, such that the total concentration of said lipid in said medium is from about 0.1% to about 30% by weight, and the ratio of lipid to surfactant is from about 5.5:1 to about 1:500, and (B) applying a suitable amount of said transfersomes in said medium onto the skin of said mammal such that an effective dose of said lipid, said surfactant, or a further medical agent associated with said transfersomes is absorbed into said mammal.

2. The method of claim 1, wherein said transfersome includes one or several layers.

3. The method of claim 1, wherein the edge tension of a transfersome is about 10 Piconewton or less.

4. The method of claim 1, wherein the concentration of said surfactant edge active substance is between 20 and 50 mol-% of the concentration of surfactant that causes the lipid to be solubilized, and the edge tension of a transfersome is about 10 Piconewton or less.

5. The method of claim 1, further comprising associating a medical agent with said transfersomes said medical agent being contained in the interior of said transfersome, in an outer membrane of said transfersome, or both.

6. The method of claim 1, wherein the total concentration of said lipid in said medium is between 0.1 and 15 weight-%.

7. The method of claim 1, wherein the total concentration of said lipid in said medium is between 5 and 10 weight-%.

8. The method of claim 5, wherein said medical agent comprises a growth modulating substance for living organisms.

9. The method of claim 5, wherein said medical agent comprises at least one antidiabetic agent.

10. The method of claim 5, wherein said medical agent comprises insulin.

11. The method of claim 1 further comprising preparing said transfersomes as **unilamellar** structures.

12. The method of claim 1 further comprising preparing said transfersomes double layer structures.

13. The method of claim 1, wherein said lipid is a synthetic lipid.

14. The method of claim 1, wherein said lipid is a phospholipid.

15. The method of claim 1, wherein said lipid comprises a glyceride.

16. The method of claim 1, wherein said lipid is selected from the group consisting of glycerophospholipid, isoprenoidlipid, sphingolipid, steroid, a sulfur-containing lipid and a carbohydrate-containing lipid.

17. The method of claim 1, wherein said lipid comprises a fatty acid.

18. The method of claim 1, wherein said lipid is selected from the group consisting of **phosphatidylcholine**, phosphatidylethanolamine, phosphatidyglycerol, phosphatidylinositol, phosphatidic acid, phosphatidylserine, sphingomyeline, sphingophospholipid, glycosphingolipid, cerebroside, ceramidepolyhexoside, sulfatide, sphingoplasmalogene, a ganglioside, a glycolipid and a synthetic lipid.

19. The method of claim 1, wherein said lipid is selected from the group consisting of a dioleoyl lipid, a dilinoleyl lipid, a dilinolenyl lipid, a dilinolenoyl lipid, a diarachidoyl lipid, a dimyristoyl lipid, a dipalmitoyl lipid, a distearoyl lipid, a diacyl lipid and a dialkyl lipid.

20. The method of claim 13, containing several surfactants.

21. The method of claim 20, wherein said surfactant is selected from the group consisting of nonionic, zwitterionic, anionic and cationic surfactants.

22. The method of claim 20, wherein said surfactant is selected from the group consisting of a long-chain fatty acid, a long-chain fatty alcohol, an alkyl-trimethyl-ammonium-salt, an alkylsulfate salt, a cholate-, a deoxycholate-, a glycodeoxycholate-, taurodeoxycholate, dodecyl-dimethyl-aminoxide, decanoyl-N-methylglucamide dodecanoyl-N-methylglucamide, N-dodecyl-N, N-dimethylglycine, 3-(hexadecyldimethylammonio)-propane-sulfonate, N-hexadecyl-sulfobetaine, nonaethylene-glycoloctylphenylether, nonaethylene-dodecylether, octaethyleneglycol-isotridecylether, octaethylenedodecylether, polyethlene glycol-20-sorbitanemonolaurate, polyhydroxyethylene-cetylstearyl ether polyhydroxyethylene-4-laurylether, polyhydroxyethylene-23-laurylether, polyhydroxyethylene-8-stearate, polyhydroxyethylene-40-stearate, polyhydroxyethylene-100-stearate, polyethoxylated castor oil 40, polyethoxylated hydrated castor oil, sorbitanemonolaurate, lauryl- salts, oleoylsulfate-salts, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium elaidate, sodium linoleate, sodium laurage, nonaethylene-dodecylether, polyethylene glycol-20-sorbitane-monooleate, polyhydroxyethylene-23-laurylether, polyhydroxyethylene-40-stearate, a sorbitane phospholipid a monolaurate phospholipid and a lysophospholipid.

23. The method of claim 10, comprising 1 through 500 I.U. insulin/ml as said medical agent.

24. The method of claim 10, comprising between 20 and 100 I.U. insulin/ml as said medical agent.

25. The method of claim 21, wherein the concentration of said lipid in said medium is between 0.1 through 20 weight-%.

26. The method of claim 21, wherein the concentration of said lipid in said medium is between 0.5 and 15 weight-%.

27. The method of claim 21, wherein the concentration of said lipid in said medium is between 2.5 and 10 weight-%.

28. The method of claim 1, comprising a **phosphatidylcholine** or phosphatidylglycol as said lipid.

29. The method of claim 1, wherein said surfactant is selected from the group consisting of lysophosphatidic acid, lysophosphoglycerol, deoxycholate, glycodeoxycholate, laurate, myristate, oleate, palmitoleate, phosphate salts thereof, sulfate salts thereof, a Tween-surfactant and a Myrj-surfactant.

30. The method of claim 23, wherein the medical agent is recombinant human insulin.

31. The method of claim 1, wherein the radius of said transfersomes is between approximately 50 and approximately 200 nm.

32. The method of claim 1, wherein the radius of said transfersomes is between approximately 100 and approximately 180 mn.

33. The method of claim 1, wherein the radius of said transfersomes is from about 50 to about 340 nm.

34. The method of claim 1, wherein the ratio of lipid to surfactant is from about 5:1 to about 1:5.

35. The method of claim 1, wherein the agent is selected from the group consisting of an adrenocorticosteroid or its analogues, an androgen, an antiandrogen, an anabolic steroid, an anaesthetic, an analgesic, an antiallergic, an antiarrhythmic, an antiarterosclerotic, an antiasthmatic, an antidepressant, an antipsychotic, an antidiabetic, an antidote, an antiemetic, an antifibrinolytic, an anticonvulsant, an anticholinergic, an enzyme, a coenzyme, an enzyme inhibitor, an antihistaminic, an antihypertonic, an anticoagulant, an antimycotic, an anti-parkinson agent, an antiphlogistic, an antipyretic, an antirheumatic, an antiseptic, a respiratory agent, a chemotherapeutic, a coronary dilator, an antineoplastic, a diuretic, a ganglium-blocker, a glucocorticoid, an immunologically active substance, a contraceptive, a morphine-antagonist, a muscle relaxant, a narcotic, a nucleotide, a neurotransmitter, an ophthalmic, a sympaticomimetic, a sympatholytic, a parasympaticomimetic, a parasympatholytic, a protein, a protein derivative, an anti-psoriatic, a psychostimulant, a sleep-inducing agent, a sedating agent, a spasmolytic, atuberculosis preparation, a vasoconstrictor, a vasodilator, a wound-healing substance and a combination thereof.

AI US 1992-844664 19920408 (7) <--

SUMM Methods deviating from the ones already described have brought little improvement to date. The use of lipoidal carriers, the **liposomes**, on intact skin, which has been theoretically discussed by several authors, was mainly aimed at modifying the agent's pharmacokinetics (Patel,. . . Sci.F.I.P.) 345-58 Elsevier, Amsterdam, 1985). Thus far, all proposal of this kind, moreover, involved the use of standard lipid vesicles (**liposomes**) which cannot penetrate the skin at all or permeate through the skin very inefficiently, as is shown in this patent application. Patent applications nos. JP 61/271204 A2 [86/271204] refer to a related use of **liposomes** in which hydrochinonglucosidal is employed to improve the stability of the agent.

SUMM . . . obtaining results which were dramatically better than those of Gesztes and Mezei. Our carrier formulations consisted of filtered lipid vesicles (**liposomes**) which also contained some detergents, with a

around 4/1.

SUMM . . . 26 834.9-41 which also refers to German patent application P 40 26 833.0-43; the latter deals with the problem of **liposome** fabrication.

SUMM . . . sufficiently elastic to penetrate through the constrictions in a barrier, such as skin. In the case of transfersomes consisting of **phosphatidylcholine** and sodium cholate this condition is fulfilled when the edge tension of a carrier is below 10 Piconewton; similar values. . .

DETD The transfersomes according to this invention differ from the **liposomes** hitherto described for topical application and from other related carriers in at least three basic features. Firstly, they can consist. . . arbitrary fashion: their penetration capacity does not depend on the manufacturing procedure. Thirdly, the penetration capability of the previously described **liposomes** optimized for applications on skin (cf. patent application P 40 26 834.9-41) was based on the use of a carrier. . .

DETD . . . and R_2 is hydroxyacyl, R_3 is a hydrogen atom and R_4 is a 2-trimethylammonioethyl (the last compound corresponding to the **phosphatidylcholine** headgroup), 2-dimethylammonioethyl, 2-methylammonioethyl or 2-aminoethyl (corresponding to a phosphatidylethanolamine headgroup).

DETD A lipid of this kind is, for example, **phosphatidylcholine** from natural sources, in the old nomenclature also called lecithin. This can be obtained, for example, from eggs (then being. . .

DETD . . . production of immunoglobulines or other immunologically active substances (endotoxines, cytokines, lymphokines, prostaglandines, leucotrienes, other immuno modulators or biological messengers), including **vaccines**. Antibodies against any of these substances can also be used; preferred are immunotransfersomes with or without endotoxines, cytokines, prostaglandines, leucotrienes,. . .

DETD . . . Hippuryl-Arg (Hip-Arg), Hippuryl-Gly-Gly (Hip-Gly-Gly), Hippuryl-His-Leu (Hip-His-Leu), Hippuryl-Lys, Hippuryl-Phe, hirudine and its fragments, His-Ala, His-Gly, His-Leu, His-Leu-Gly-Leu-Ala-Arg, His-Lys, His-Phe, His-Ser, His-Tyr, **HIV** envelope protein (gp120), Hydra peptides, P-hydroxyhippuryl-His-Leu, hypercalcemia malignancy factor (1-40), insulin chains B and C, P-iodo-Phe, Ile-Asn, Ile-Pro-Ile, insulin-like growth. . .

DETD . . . (1.1.1.50), 3beta-hydroxysteroid dehydrogenase (1.1.1.51), 3alpha,2beta-hydroxysteroid dehydrogenase (1.1.1.53), 3-phosphoglycerate dehydrogenase (1.1.1.95), fucose dehydrogenase (1.1.1.122), lactate dehydrogenase (cytochrome) (1.1.2.3), glucose oxidase (1.1.3.4), **cholesterol** oxidase (1.1.3.6), galactose oxidase (1.1.3.9), alcohol oxidase (1.1.3.13), glycolate oxidase (1.1.3.15), choline oxidase (1.1.3.17), glycerol-3-phosphate oxidase (1.1.3.21), xanthine oxidase (1.1.3.22),. . .

DETD Transpeptidases, such as: esterase (3.1.1.1), lipase (3.1.1.3), phospholipase A (3.1.1.4), acetylesterase (3.1.1.6), cholinesterase, acetyl (3.1.1.7), cholineesterase, butyryl (3.1.1.8), pectinesterase (3.1.1.11), **cholesterol** esterase (3.1.1.13), glyoxalase ii (3.1.2.6), phosphatase, alkaline (3.1.3.1), phosphatase acid (3.1.3.2), 5'-nucleotidase (3.1.3.5), 3'-nucleotidase (3.1.3.6), glucose-6-phosphatase (3.1.3.9), fructose-1,6-diphosphatase (3.1.3.11), phytase. . .

DETD . . . example, in our previous German patent application P 40 26 833.0-43, and exemplified in several cases in the handbook on `**Liposomes**` (Gregoriadis, G., Edits. CRC Press, Boca Raton, Fla., Vols 1-3, 1987), in the monography `**Liposomes** as drug carriers` (Gregoriadis, G., Edits. John Wiley & Sons, New York, 1988), or in the laboratory manual `**Liposomes**. A Practical Approach` (New, R., Oxford-Press, 1989). If required any suspension of drugs, moreover, can be diluted or concentrated (e.g.. . .

DETD Methods for the preparation of **liposomes**, which in the majority of cases can also be used for manufacturing transfersomes, are described, for example, in `**Liposome** Technology` (Gregoriadis, Ed., CRC Press) or older books dealing with similar topics, such as `**Liposomes** in

Systems` (Gregoriadis & Allison, Eds., Willey), `Targeting of Drugs` (Gregoriadis & Senior & Trouet, Plenum), etc. Corresponding patent. . .

DETD

250-372 mg	**phosphatidylcholine** from soy-bean (+95% = **PC**)
187-34.9 mg	oleic acid (+99%)
0.312-0.465 ml	ethanol, absolute
10 mM	Hepes

DETD Increasing amounts of oleic acid were pipetted into different volumes of alcoholic **PC**-solutions containing 75 micromoles of lipid so as to create a concentration series with a lipid/surfactant ratio beginning with L/S=0.5 and. . . M NaOH, the first incubation period was followed by another incubation for 24 hours. In order to obtain a final **liposome** suspension, each sample was thoroughly mixed and filtered through a polycarbonate filter (0.45 micrometer) into a glass vial which was. . .

DETD . . . concentration of fatty acid in the transfersomes. This trend is not monotonous, however. At a lipid/surfactant-ratio of approx. 2, the **liposome** permeation capacity starts to increase; but it then decreases again until, for L/S above 3, the transfersomes have nearly lost. . . At this concentration ratio, which corresponds roughly to 30% of the solubilization dose of fatty acids in an alkaline suspension, **liposomes** thus appear to correspond to optimal transfersomes.

DETD

349-358 mg	**phosphatidylcholine** from soy-bean (+95% = **PC**)
63.6-52.2 mg	oleic acid (+99%)
10 mM	Hepes

DETD

322.6-372 mg	phosphatidycholine from soy-bean (+95% = **PC**)
96.8-34.9 mg	oleic acid (+99%)
0.403-0.465 ml	ethanol, absolute
10 mM	Hepes
130 mM	NaCl, p.a.

DETD

184.5-199.8 mg	**phosphatidylcholine** from soy-bean (+95% = **PC**)
20.5-22.2 mg	phosphatidylglycerol from egg **PC** (puriss., Na-salt, = PG)
44.9-26.1 μl	oleic acid (+99%)
0.165-0.178 ml	ethanol, absolute
4.5 ml	Hepes, 10 mM

DETD Anhydrous PG is mixed with an alcoholic solution of **PC** to give a clear solution with 90% **PC** and 10% PG. Oleic acid is added to this solution; the resulting lipid/surfactant ratios are between 1.6 and 2.8; an. . .

DETD . . . carriers which contained no charged species but had a similar L/S-ratio. Based on our experiments with a 4% suspension of **PC** and oleic acid we conclude that the relatively low total lipid concentration plays only a minor role in this respect.

DETD As in previous examples, a resistance minimum is observed for the 4% **PC**/PG mixtures; this minimum, however, is found with L/S-ratios which are by some 20% higher than those measured with 8% lipid. . .

DETD

[illegible] mg
phosphatidylcholine from soy-bean (+95% = **PC**)
123.3-80.8 µl
Tween 80 (puriss.)
0.38-0.42 ml
ethanol, absolute
4.5 ml phosphate buffer, isotonic, sterile

DETD Increasing volumes of Tween 80 are pipetted into appropriate volumes of an alcoholic **PC** solution. This gives rise to a concentration series with 12.5 through 25 mol-% surfactant (L/S=4-8). In addition to this, samples. . .

DETD

314.2-335.4 mg
soy-bean **phosphatidylcholine** (+95% = **PC**)
107.2-80.8 ml
Tween 80 (puriss.)
4.5 ml phosphate buffer, isotonic, sterile

DETD First Tween 80 and subsequently phosphate buffer are added to appropriate quantities of **PC**. The resulting mixture is agitated at room temperature for 4 days. The further procedure is as described in examples 40-49.

DETD

193-361 mg
phosphatidylcholine from soy-bean (grade I, S100)
207.2-38.8 mg
Na-cholate, puriss.
4.5 ml phosphate buffer (isotonic with a physiologic solution)
ethanol, absolute

DETD

1.627-0.5442 g
phosphatidylcholine from soy-bean (gradeI, S100)
4.373-0.468 g
Na-cholate, puriss.
60 ml phosphate buffer (physiological)

DETD

16.3-5.4 mg **phosphatidylcholine** from soy-bean (Grade I, S100)
41.5-5.5 mg Na-desoxycholate, puriss.
5 ml phosphate buffer (physiological)

DETD

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3 mM Suspension of **phosphatidylcholine** from soy-bean (grade I, S100) in phosphate buffer Na-cholate,

---

puriss.

DETD . . . this series 1 cholate molecule per lipid is required for a rapid formation of vesicles (for the formation of largely **unilamellar** vesicles).

DETD

121.2-418.3 mg
phosphatidylcholine from soy-bean (Grade I,
PC) 378.8-81.7 mg
Triton X-100
4.5 ml 0.9% NaCl solution in water

DETD A 10% **PC**-suspension in isotonic solution of sodium chloride is homogenized at 22° C. until the mean size of lipid vesicles is approx.. . . A sufficient volume of Triton X-100 is pipetted into each of these aliquots to give a concentration series with nominal **PC**/Triton ratios in the range of 0.25 through 4 in steps of 0.5. All resulting samples are occasionally mixed and incubated. . .

DETD . . . solubilization, shall be represented in the right panel of FIG. 8. The solubilization limit is approx. 2 triton molecules per **PC**-molecule. Right below this limit, the optical density (OD (400 nm))--and thus the vesicle diameters--attain the greatest values. At **PC**/triton ratios higher than 2,5/1, the change in the optical density of given suspensions is only minimal.

DETD **Liposomes** were pressed through a 0.2 micrometer filter. Simultaneously, the permeation resistance was measured. Vesicles with an L/S ratio below 4/1. . .

DETD

101.6-227 mg **phosphatidylcholine** from soy-bean
148.4-22.2 mg octyl-glucopyranoside (β-octylglucoside),
puriss. 9.85 ml
phosphate buffer, pH 7.3
ethanol, absolute

DETD **Phosphatidylcholine** in ethanol (50%) and octylglucopyranoside were mixed in different relative ratios in order to prepare a concentration series with increasing. . .

DETD

43.3 mg, 50 mg
phosphatidylcholine from soy-bean
0.5 mg phosphatidylethanolamine-N-fluorescein
6.7 mg, 0 mg cholate, Na-salt, p.a.
5 ml Hepes-buffer, pH 7.3

DETD **Phosphatidylcholine** with the addition of 1%-fluoresceinated lipids with or without desoxycholate is suspended in 5 ml buffer. The lipid/surfactant ratio is. . .

DETD

43.5, 45.3, 50 mg
phosphatidylcholine from soy-bean
0.5 mg phosphatidylethanolamine-N-fluorescein
6.5, 4.7, 0 mg
desoxycholate, Na-salt, p.a.
25 ml Hepes-buffer, pH 7,3

DETD

50 mg; 43.3 mg, 15.9 mg
phosphatidylcholine from soy-bean
0.5mg phosphatidylethanolamine-N-fluorescein
0 mg; 6.7 mg; 34.1 mg
cholate, Na-salt, p.a.
5 ml Hepes-buffer, pH 7.3

DETD Lipid vesicles consisting of **phosphatidylcholine** and a fluorescent additive were made as in examples 137-138. For this experiment, suspensions with a lipid/surfactant ratio of 1/0,. . .

DETD

50 mg; 43.5 mg; 17.1 mg
phosphatidylcholine from soy-bean
0.5 mg phosphatidylethanolamine-N-fluorescein
0 mg; 4.7 mg; 32.9 mg
desoxycholate, Na-salt, p.a.
5 ml Hepes-buffer, pH 7.3

DETD

50 mg; 36.4; 20 mg
phosphatidylcholine from soy-bean
0.5 mg phosphatidylethanolamine-N-fluorescein
0 mg; 13.6 mg; 30 mg
Brij 35
5 ml Water

DETD

	phosphatidylcholine from soy-bean 80%
75 kBq	Giberellin A4, 3H-labelled
15.8 to 75 mg	
	polyoxyethylene (23)-laurylether (Brij 35)
1 ml	water
	ethanol, absolute

DETD

32.8-0.64 mg	**phosphatidylcholine** from soy-bean (purity higher than 95%, **PC**)
75 kBq	dipalmitoylphosphatidylcholine tritium-labelled
2.2-34.4 mg	bile acid, Na-salt, p.a.
0.32 ml	phosphate buffer, pH 7.3

DETD . . . For comparison, the normalized values are also given which were taken from our patent application pertaining to the use of **liposomes** for topical anaesthesia. Optimal transfersomes are appreciably better than non-optimal preparations containing surfactants.

DETD

31 mg	**phosphatidylcholine** from soy-bean (purity higher than 95%, **PC**)
75 kBq	dipalmitoylphosphatidylcholine tritium-labelled
4 mg	deoxycholate, Na-salt, p.a.
0.32 ml	phosphate buffer, pH 7.3

DETD In each case 35 mg of lipid (**PC** and deoxycholate) are mixed with tritium-labelled dipalmitoylphosphatidylcholine in a chloroform solution. The resulting lipid mixture is dried and then dissolved. . .

DETD Experimental data show (FIG. 11) that systemically applied transfersomes are eliminated from blood comparably as rapidly as standard **liposomes**. The size of carrier particles appears not to affect the spontaneous penetration into skin. All transfersomes investigated in this study. . .

DETD

88 mg	**phosphatidylcholine** from soy-bean (purity higher than 95%, **PC**)
75 kBq	insulin, tritium labelled
12 mg	deoxycholate, Na-salt, p.a.
100 ml	ethanol, absolute
0.9 ml	isotonic salt solution

DETD 100 mg of **PC** dissolved in 100 ml of warm ethanol, or a corresponding **PC**/deoxycholate solution (L/S=4.5), are mixed with 0.9 ml of an isotonic salt solution (suspensions A and B, respectively). Each suspension is ultrasonicated until the mean vesicle size is about **150 nm**.

DETD . . . microliters of an aqueous solution of tritium-labelled inulin are pipetted into 38 microliters of a freshly prepared suspension of empty **liposomes** (A) or transfersomes (B). Subsequently, all mixtures are sonicated in closed vials for 60 minutes in an ultrasound bath at. . .

DETD The results of this study are collected in FIG. 12. They show that normal **liposomes** can hardly mediate a percutaneous inulin uptake; in contrast to this, 6 hours later approx. 1.4% of this marker which. . .

DETD In contrast to this, approximately 2% of the normal **liposomes** at the application site can be detected by eye, the corresponding doses in the liver and spleen being below 0.1%.. . .

DETD

386 mg	**phosphatidylcholine** from soy-bean (purity > 95%)
58.5 mg	sodium-cholate (L/S = 3.5)
500 ml	ethanol (96%)
2.25 ml	0.9% NaCl solution (per inject.)

DETD . .

956 mg **phosphatidylcholine** from soy-bean (+95%)
0-26 mg sodium-deoxycholate
1 mg prostaglandine E1
1 ml ethanol absolute
50 ml 0.9% NaCl solution (per inject.)

DETD This shows that merely transfersomes--but not **liposomes** or sub-optimal surfactant-containing vesicles--can penetrate into intact skin and thereby transfer drugs into body. The precise mode of sample preparation. . .

DETD

79.4 mg; 88.5 mg **phosphatidylcholine** from soy-bean (+95%)
20.6 mg, 11.5 mg sodium-deoxycholate
10 µg hydrocortison
0.1 ml ethanol absolute
1 ml phosphate buffer, physiological

DETD

1.1-2 mg **phosphatidylcholine** from soy-bean (+95% = **PC**)
0-32.5 mol-% Tween 80
pH 7.2 isotonic phosphate buffer

DETD

256.4-447 mg **phosphatidylcholine** from soy-bean (+95% **PC**)
243.6-53.1 mg Brij 96
0.26-0.45 ml ethanol, absolute
4.5 ml phosphate buffer, pH 6.5. 10 mM

DETD Increasing volumes of Brij 96 are pipetted into the corresponding volumes of an alcoholic **PC** solution. Thus, a concentration series is obtained with L/S values between 1/1 and 1/8. After the addition of a buffer very heterogeneous **liposomes** are formed which are homogenized by means of filtering through a 0.2 µm filter.

DETD

202.0-413 mg **phosphatidylcholine** from soy-bean (+95% = **PC**)
298.0-87.0 mg Myrj 49
0.26-0.45 ml ethanol, absolute
4.5 ml phosphate buffer, pH 6.5. 10 mM

DETD

144.9 mg **phosphatidylcholine** from soy-bean
24.8 mg desoxycholate, Na-salt
1.45 ml Actrapid HM 100 (145 I.U.)
0.16 ml ethanol, absolute

DETD These **liposomes** with a relatively high surfactant concentration have only a very limited capability of transporting insulin across skin, as is seen. . . of 30-40 minutes at the most. The effect of a comparable subcutaneous injection is 50 to 200 times higher. Surfactant-containing **liposomes**, which have not been optimized with regard to their `transfersomal` properties, are consequently poorly suited for the use as carriers. . .

DETD . . . et al., J. Pharmacobio.-Dynam. 12, 31, 1989), perocularly (Chiou et al., J. Ocul. Pharmacol. 5, 81, 1989), perorally in a **liposomes** suspension (Rowland & Woodley, Biosc. Rep. 1, 345, 1981) or

[illegible] skin,. . .

DETD . . . for delayed insulin release in insulin implants (Wang, P. Y Int. J Pharm. 54, 223, 1989); in the form of **liposomes** they were also suggested for use as vehicles for peroral applications (Patel, 1970), without the therapeutic results really being reproducible, however, (Biochem. Int. 16, 983, 1988). Subsequent publications in the field of insulin containing **liposomes**, therefore, have dealt with methodological rather than therapeutic issues (Wiessner, J. H. and Hwang, K. J. Biochim. Biophys. Acta 689,. . .

DETD

120 mg	**phosphatidylcholine** from soy-bean (purity > 95%)
20 mg	sodium-cholate p.a. (L/D = 3.2)
150 µl	ethanol (96%)
1.45 ml	Actrapid HM 100 (recombinant human. . .

DETD

216 mg	**phosphatidylcholine** from soy-bean (487 µl of a 50% solution in absolute ethanol)
27 mg	phosphatidylglycerol from egg (98%)
29.45 mg	oleic acid, puriss.

3. . .

DETD

143 mg	**phosphatidylcholine** from soy-bean
18 mg	phosphatidylglycerol from egg (98%)
19.6 mg	oleic acid, puriss.
2 ml	Actrapid HM 100 (200 I.U.)
25 µl	1N NaOH

DETD

143 mg	**phosphatidylcholine** from soy-bean
18 mg	phosphatidylglycerol from egg (98%)
20.5 mg	sodium oleate
2 ml	Actrapid HM 100 (200 I.U.)

DETD

144.9; 152 mg	**phosphatidylcholine** from soy-bean
24.8; 17.6 mg	desoxycholate, Na-salt
1.45; 1.55 ml	Actrapid HM 100 (145 I.U.)
0.16 ml	ethanol, absolute

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11. The method of claim 1 further comprising preparing said transfersomes as **unilamellar** structures.

18. The method of claim 1, wherein said lipid is selected from the group consisting of **phosphatidylcholine**, phosphatidylethanolamine, phosphatidyglycerol, phosphatidylinositol, phosphatidic acid, phosphatidylserine, sphingomyeline, sphingophospholipid, glycosphingolipid, cerebroside, ceramidepolyhexoside, sulfatide, sphingoplasmalogene, a ganglioside, a glycolipid and a synthetic. . .

28. The method of claim 1, comprising a **phosphatidylcholine** or phosphatidylglycol as said lipid.

L27 ANSWER 3 OF 8 USPATFULL on STN

1999:136717 Lipid-nucleic acid particles prepared via a hydrophobic lipid-nucleic acid complex intermediate and use for gene transfer.

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US 5976567 19991102

DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Novel lipid-nucleic acid particulate complexes which are useful for in vitro or in vivo gene transfer are described. The particles can be formed using either detergent dialysis methods or methods which utilize organic solvents. Upon removal of a solubilizing component (i.e., detergent or an organic solvent) the lipid-nucleic acid complexes form particles wherein the nucleic acid is serum-stable and is protected from degradation. The particles thus formed have access to extravascular sites and target cell populations and are suitable for the therapeutic delivery of nucleic acids.

CLM What is claimed is:

1. A method for the preparation of lipid-nucleic acid particles, comprising: (a) contacting nucleic acids with a solution comprising non-cationic lipids and a detergent to form a nucleic acid-lipid mixture; (b) contacting cationic lipids with said nucleic acid-lipid mixture to neutralize the negative charge of said nucleic acids and form a charge-neutralized mixture comprising detergent, nucleic acids and lipids; and (c) removing said detergent from said charge-neutralized mixture to provide said lipid-nucleic acid particles in which said nucleic acids are protected from degradation.

2. The method in accordance with claim 1, wherein said solution of step (a) further comprises an organic solvent.

3. The method in accordance with claim 1, wherein said cationic lipids are members selected from the group consisting of DODAC, DDAB, DOTMA, DOSPA, DMRIE, DOGS and combinations thereof.

4. The method in accordance with claim 1, wherein said non-cationic lipids are selected from the group consisting of ESM, DOPE, polyethylene glycol-based polymers and combinations thereof.

5. The method in accordance with claim 1, wherein said detergent is octyl-β-D-glucopyranoside, said cationic lipid is DODAC, said non-cationic lipid is ESM, and said detergent is removed by dialysis.

6. The method in accordance with claim 5, wherein said non-cationic lipids are combinations of ESM and PEG-Ceramide.

7. The lipid-nucleic acid particle prepared according to claim 1.

8. A method for the preparation of lipid-nucleic acid particles, comprising: (a) contacting an amount of cationic lipids with nucleic acids in a solution; said solution comprising of from about 15-35% water and about 65-85% organic solvent and said amount of cationic lipids being sufficient to produce a +/- charge ratio of from about 0.85 to about 2.0, to provide a hydrophobic, charge-neutralized lipid-nucleic acid complex; (b) contacting said hydrophobic lipid-nucleic acid complex in solution with non-cationic lipids, to provide a lipid-nucleic acid mixture; and (c) removing said organic solvents from said mixture to provide said lipid-nucleic acid particles in which said nucleic acids are protected from degradation.

9. The method in accordance with claim 8, wherein said cationic lipids are members selected from the group consisting of DODAC, DDAB, DOTMA, DOSPA, DMRIE, DOGS and combinations thereof.

10. The method in accordance with claim 8, wherein said non-cationic lipids are members selected from the group consisting of ESM, DOPE, polyethylene glycol-based polymers and combinations thereof.

11. The method in accordance with claim 8, wherein said organic solvents are members selected from the group consisting of methanol, chloroform, methylene chloride, ethanol, diethyl ether and combinations thereof.

12. The method in accordance with claim 8, wherein said nucleic acid is a plasmid, said cationic lipid is a member selected from the group consisting of DODAC, DDAB, DOTMA, DOSPA, DMRIE, DOGS and combinations thereof said non-cationic lipid is a member selected from the group consisting of ESM, DOPE, polyethylene glycol-based polymers and combinations thereof, and said organic solvent is a member selected from the group consisting of methanol, chloroform, methylene chloride, ethanol, diethyl ether and combinations thereof.

13. The lipid-nucleic acid particle prepared according to claim 8.

14. The method for the preparation of serum-stable plasmid-lipid particles, comprising: (a) combining a plasmid with cationic lipids in a detergent solution to provide a coated plasmid-lipid complex; (b) contacting non-cationic lipids with said coated plasmid-lipicl complex to provide a solution comprising detergent, a plasmid-lipid complex and non-cationic lipids; and (c) removing said detergent from said solution of step (b) to provide a solution of serum-stable plasmid-lipid particles, wherein said plasmid is encapsulated in a lipid bilayer and said particles are serum-stable and have a size of from about 50 to about **150 nm**.

15. The method in accordance with claim 14, wherein said removing is by dialysis.

16. The method in accordance with claim 14, wherein step (b) further comprises adding a polyethylene glycol-lipid conjugate.

17. The method in accordance with claim 14, wherein said polyethylene glycol-lipid conjugate is a PEG-ceramide conjugate.

18. The method in accordance with claim 14, further comprising; (d) sizing said particles to achieve a uniform particle size.

19. The method in accordance with claim 14, wherein said cationic lipids are selected from the group consisting of DODAC, DDAB, DOTAP, DOTMA, DOSPA, DOGS, DC-Chol and combinations thereof.

20. The method in accordance with claim 14, wherein said non-cationic lipids are selected from the group consisting of DOPE, POPC, EPC and combinations thereof.

21. The method in accordance with claim 14, wherein said detergent solution comprises a detergent having a critical micelle concentration of between about 20 mM and 50 mM.

22. The method in accordance with claim 21, wherein said detergent is n-octyl-β-D-glucopyranoside.

23. The plasmid-lipid particle prepared according to claim 14.

24. A method for the preparation of serum-stable plasmid-lipid particles, comprising; (a) preparing a mixture comprising cationic lipids and non-cationic lipids in an organic solvent; (b) contacting an aqueous solution of plasmid with said mixture prepared in step (a) to provide a clear single phase; and (c) removing said organic solvent to provide a suspension of plasmid-lipid particles, wherein said plasmid is encapsulated in a lipid bilayer, and said particles are stable in serum and have a size of from about 50 to about **150 nm**.

25. The method in accordance with claim 24, wherein said non-cationic lipids comprise a polyethylene glycol-lipid conjugate.

26. The method in accordance with claim 25, wherein said polyethylene glycol-lipid conjugate is a PEG-ceramide conjugate.

27. The method in accordance with claim 24, wherein said cationic lipids are selected from the group consisting of DODAC, DDAB, DOTAP, DOTMA, DOSPA, DOGS, DC-Chol and combinations thereof.

28. The method in accordance with claim 24, wherein said nori-cationic lipids are selected from the group consisting of DOPE, POPC, EPC and combinations thereof.

29. The plasmid-lipid particle prepared according to claim 24.

AI US 1996-660025 19960606 (8) <--

SUMM . . . lipids (see Leventis, et al., Biochem. Biophys. Acta 1023:124 (1990); lipopolyamines (see, Behr, et al., U.S. Pat. No. 5,171,678) and **cholesterol** based lipids (see Epand, et al., WO 93/05162, and U.S. Pat. No. 5,283,185). It has been reported that DOTMA and. . .

SUMM . . . transfer have generally been formulated in one of two ways. In one method, the nucleic acid is introduced into preformed **liposomes** made of mixture of cationic lipids and neutral lipids. The complexes thus formed have undefined and complicated structures and the. . .

SUMM . . . particles, in which the plasmid is encapsulated in a lipid bilayer. The particles thus formed have a size of about 50-**150 nm**.

SUMM . . . plasmid is encapsulated in a lipid bilayer, and the particles are stable in serum and have a size of about 50-**150 nm**.

DRWD FIGS. 12A and 12B illustrate the resistance of plasmid complexed to preformed **liposomes** composed of DOPE:DODAC(50:50) (A) and plasmid encapsulated within DOPE:DODAC:PEG-Cer-C_{14} particles (B) to digestion by DNAse I. Plasmid DNA was extracted. . .

DRWD FIGS. 17A and 17B provide electron micrographs of **liposomes** composed of DOPE:DODAC:PEO-Cer-C_{20} without encapsulated plasmid (A) and the plasmid:lipid particles (B). The small arrows denote empty **liposomes** approximately 100 nm in diameter. These are compared to electron-dense particles surrounded by a membrane bilayer (large arrows). Scale bar=100. . .

DETD The following abbreviations are used herein: CHO, Chinese hamster ovary cell line; B16, murine melanoma cell line; DC-Chol, 3β-(N-(N',N'-dimethylaminoethane)carbamoyl) **cholesterol** (see, Gao, et al., Biochem. Biophys. Res. Comm. 179:280-285 (1991)); DDAB, N,N-distearyl-N,N-dimethylammonium bromide; DMRIE, N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide; DODAC, N,N-dioleyl-N,N-dimethylammonium chloride; DOGS, diheptadecylamidoglycyl spermidine; DOPE, 1,2-sn-dioleoylphoshatidylethanolamine; DOSPA, N-(1-(2, 3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate; DOTAP, N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; DOTMA, N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammoniumchloride; EPC, egg **phosphatidylcholine**; ESM, egg sphingomyelin; RT, room temperature; TBE, Tris-Borate-EDTA (89 mM in Tris-borate and 2 mM in EDTA); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;. . . PEG-Cer-C_{14}, 1-O-(2'-(ω-methoxypolyethyleneglycol)succinoyl)-2-N-myristoyl-sphingosine; PBS, phosphate-buffered saline; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; OGP, n-octyl β-D-glycopyranoside (Sigma Chemical Co., St. Louis, Mo.); POPC, palmitoyl oleoyl **phosphatidylcholine** (Northern Lipids, Vancouver, BC); QELS, quasielastic light scattering; TBE, 89 mM Tris-borate with 2 mM EDTA; and EDTA, Ethylenediaminetetraacetic acid. . .

DETD . . . by one or more aromatic, cycloaliphatic or heterocyclic group(s). Preferred lipids are phosphoglycerides and sphingolipids, representative examples of which include **phosphatidylcholine**, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl **phosphatidylcholine**, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine or dilinoleoylphosphatidylcholine could be used. Other compounds lacking in phosphorus, such as sphingolipid and

glycosphingolipid. . .

DETD . . . cationic lipids are available which can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic **liposomes** comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE® (commercially available cationic **liposomes** comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising DOGS from Promega Corp., Madison, Wis., USA).

DETD Gene transfer techniques that involve the use of **liposomes** have been described previously in the art (U.S. Pat. Nos. 5,049,386; 4,946,787; and 4,897,355). General lipofection protocols are also described. . .

DETD . . . cationic lipids are available and can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic **liposomes** comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE® (commercially available cationic **liposomes** comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic **liposomes** comprising DOGS from Promega Corp., Madison, Wis., USA).

DETD . . . include: phospholipid-related materials, such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, dioleoylphosphatidylcholine (DOPC), dipalmitoyl-**phosphatidylcholine** (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoy-lphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal). Additional non-phosphorous containing lipids are,. . .

DETD . . . murine cytomegalovirus, the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and **HIV**. See, Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by reference.

DETD Plasmids designed for producing recombinant **vaccinia**, such as pGS62, (Langford, C. L. et al. (1986), Mol. Cell. Biol., 6: 3191-3199) may also be used. This plasmid consists of a cloning site for insertion of foreign nucleic acids, the P7.5 promoter of **vaccinia** to direct synthesis of the inserted nucleic acid, and the **vaccinia** TK gene flanking both ends of the foreign nucleic acid.

DETD Lipid-nucleic acid formulations can be formed by combining the nucleic acid with a preformed cationic **liposome** (see, U.S. Pat. Nos. 4,897,355, 5,264,618, 5,279,833 and 5,283,185). In such methods, the nucleic acid is attracted to the cationic surface charge of the **liposome** and the resulting complexes are thought to be of the **liposome**-covered "sandwich-type." As a result, a portion of the nucleic acid or plasmid remains exposed in serum and can be degraded. . . enzymes such as DNAse I. Others have attempted to incorporate the nucleic acid or plasmid into the interior of a **liposome** during formation. These methods typically result in the aggregation in solution of the cationic lipid-nucleic acid complexes (see FIG. 2). Passive loading of a plasmid into a preformed **liposome** has also not proven successful. Finally, the **liposome**-plasmid complexes which have been formed are typically 200 to 400 nm in size and are therefore cleared more rapidly from. . .

DETD . . . vitro applications the particles are more preferably negatively charged. This provides the further advantage of reduced aggregation over the positively-charged **liposome** formulations in which a nucleic acid can be encapsulated in cationic lipids.

DETD The particles made by the methods of this invention have a size of about 50 to about **150 nm**, with a majority of the particles being about 65 to 85 nm. The particles can be formed by either a. . . in which the plasmid is encapsulated in a lipid bilayer. As noted above, these particles differ from the more classical **liposomes** both in size (**liposomes** being typically 200-400 nm) in that there is little or no

aqueous medium encapsulated by the particle's lipid bilayer. The. . .

DETD . . . encapsulated in a lipid bilayer and the particles are serum-stable and have a size of from about 50 to about **150 nm**.

DETD . . . are lauroyl, myristoyl, palmitoyl, stearoyl or oleoyl. In particularly preferred embodiments, the non-cationic lipid will be 1,2-sn-dioleoylphosphatidylethanolamine (DOPE), palmitoyl oleoyl **phosphatidylcholine** (POPC) or egg **phosphatidylcholine** (EPC). In the most preferred embodiments, the plasmid-lipid particles will be fusogenic particles with enhanced properties in vivo and the. . .

DETD . . . lipid-bilayer which surrounds the plasmid providing serum-stable plasmid-lipid particles which have a size of from about 50 nm to about **150 nm**. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

DETD The serum-stable plasmid-lipid particles can be sized by any of the methods available for sizing **liposomes**. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

DETD Several techniques are available for sizing the particles to a desired size. One sizing method, used for **liposomes** and equally applicable to the present particles is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a. . .

DETD . . . a lipid bilayer, and said particles are stable in serum and have a size of from about 50 to about **150 nm**.

DETD The serum-stable plasmid-lipid particles thus formed will typically be sized from about 50 nm to **150 nm**. To achieve further size reduction or homogeneity of size in the particles, sizing can be conducted as described above.

DETD . . . cationic lipids are available and can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic **liposomes** comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE® (commercially available cationic **liposomes** comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising DOGS in ethanol from Promega Corp., Madison,. . .

DETD . . . internalization and/or membrane fusion include the gB, gC, gD, gE, gH, and gI virion glycoproteins of HSV-1, and gp120 of **HIV**-1.

DETD . . . which can be maintained from 8, 12, or up to 24 hours in the bloodstream are particularly preferred. Negatively charged **liposomes** and particles are typically, taken up more rapidly by the reticuloendothelial system (Juliano, Biochem. Biophys. Res. Commun. 63:651 (1975)) and. . .

DETD . . . fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using **liposomes**. Brigham, et al., Am. J. Med. Sci. 298:278-281 (1989), incorporated herein by reference, describes the in vivo transfection of lungs. . .

DETD . . . therapeutic treatment of patients or cells, infected with or at risk of being infected with, a pathogenic microorganism, such as **HIV**. The effectiveness of antisense molecules in blocking target gene functions of impeding virus replication has been demonstrated in a number. . . from the genome of the natural gencme of a patient undergoing therapy). For example, suitable sites for inhibition on the **HIV** virus includes TAR, REV or nef (Chatterjee et al., Science 258, 1485-1488 (1992)). Rev is a regulatory RNA binding protein that facilitates the export of unspliced **HIV** pre mRNA from the nucleus. Malim et al., Nature 338, 254 (1989). Tat is thought to be a transcriptional activator. . . cells, either ex vivo or by intravenous injection in a therapeutically effective dose. The treatment can be administered prophylactically to **HIV**⁻ persons, or to persons already infected with **HIV**.

DETD . . . Sci. USA 91: 8915-8919 (1994); Alexander et al., J. Virol. 68: 8282-8287 (1994); Srivastrava, Blood Cells 20: 531-538 (1994)). Recently, **HIV**-based vectors has been reported to transfect non-dividing cells (CITE) Nonetheless, the majority of stem cells, a preferred target for many. . .

DETD . . . extrusion 5 times through one 200 nm filter. The resulting

vesicle size was approximately 100 to 200 nm in diameter. **Liposome** sizes before extrusion varied greatly depending on the lipid composition.

DETD Cryoelectron microscopy is a relatively nonperturbing technique which routinely has been used to study **liposome** shape. **Liposomes** are visible in the vitreous ice layer due to the relatively electron-dense phosphate head groups. The same would apply to. . .

DETD . . . this preparation, 400 μg of plasmid DNA was used. The lipid composition was DOPE:DODAC:PEG-Cer-C_{14} (84:6:10). The small arrows denote empty **liposomes** approximately 100 nm in diameter. These are compared to the lipid particles containing electron-dense centers (large arrows). These electi-on-dense centers. . .

DETD LIPOFECTIN® consists of sonicated **unilamellar** vesicles composed of DOTMA and DOPE (50:50 mole ratio, see, Felgner, et al., Proc. Natl. Acad. Sci, USA 84:7413-7417 (1987)). The **liposomes** are prepared in water and are provided at a total lipid concentration of 1 mg/mL. DNA (10 μg) was mixed with the **liposomes** in water, as described below in Example 2, to provide from 0 to 160 nmoles total lipid. Each of the. . . DNA resulted in greater than 95% loss of DNA from the aqueous phase. This effect could not be achieved using **liposomes** prepared from egg **phosphatidylcholine**/DOPE (50:50 mole ratio). Thus, the hydrophobic complex which forms and is drawn into the organic phase is a result of. . .

DETD LIPOFECTAMINE® (DOSPA:DOPE, 75:25 mol ratio), and TRANSFECTAM® (100% DOGS) were added to DNA (10 μg) as preformed **liposomes**, as described in Example 13. The **liposomes** contain headgroups derived from spermine and exhibit positive charges of 5 and 4, respectively at pH <7. As expected, significantly. . .

DETD . . . reduced mobility in an agarose gel. This result is consistent with studies which have demonstrated that DNA condensed with cationic **liposomes** adopt a macromolecular structure that does not move within an applied electric field (see, Bertling, et al., Biotechnol. Appl. Biochem.. . .

DETD . . . wavelength of 632.8 nm). FIG. 31 shows that two populations of particles were observed, one group sized from 50 to **150 nm** and the second sized 500 to 1000 nm. The relative numbers of each depended on the type of non-cationic lipid(s). . .

DETD . . . nucleic acid-lipid particles could be monitored. This technique was also used to assess the ability of OGP to solubilize preformed **liposomes** of DODAC or SM. Multilamellar **liposomes** were prepared at a final lipid concentration of 1.0 mM by hydrating powdered lipid in distilled water at 60° C.. . .

DETD . . . nucleic acid-lipid particle formation in the presence of 20 mM OGP is likely not due to DNA-mediated aggregation of cationic **liposomes**. We believe that nucleic acid-lipid particle formation is the result of the hydrophobic lipid-DNA complex adopting a structure that minimizes. . .

DETD . . . bilayer structures. Instead, numerous bumps were detected on the replica. This is consistent with the suggestion that particles rather than **liposomes** were formed using the procedures described here.

DETD . . . the cationic lipid to DNA nucleotide phosphate (charge) ratio increased from 1:1 to 4:1 (FIG. 39A). Unlike results with preformed **liposome**-DNA aggregates (see, e.g., Jarnagin et al., 1992): however, transfection was not affected by the presence of serum.

DETD A significant difference between preformed **liposome**-DNA aggregates and the nucleic acid-lipid particles concerns the use of DOPE as a helper lipid required for optimal transfection (Felgner. . .

. . . encapsulated in a lipid bilayer and said particles are serum-stable and have a size of from about 50 to about **150 nm**.

. . . a lipid bilayer, and said particles are stable in serum and have a size of from about 50 to about **150 nm**.

L27 ANSWER 4 OF 8 USPATFULL on STN

1999:30374 Immunostimulating and immunopotentiating reconstituted influenza

virosomes and vaccines containing them.
Gluck, Reinhard, Spiegel/Bern, Switzerland
Mischler, Robert, Worblaufen, Switzerland
Schweiz, Serum- & Impfinstitut Bern, Bern, Switzerland (non-U.S. corporation)
US 5879685 19990309
APPLICATION: US 1994-225740 19940411 (8) <--
PRIORITY: EP 1991-107527 19910508
EP 1991-107647 19910510
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB Immunostimulating reconstituted influenza virosomes (IRIVs) are provided wherein an antigen or a combination of antigens are incorporated into a virosome further containing a mixture of phospholipids, an essentially reconstituted functional virus envelope, and influenza hemagglutinin protein (HA). The HA induces fusion of the IRIV with cellular membranes and thereby induces lysis of the IRIV after its endocytosis by antigen presenting cells.
CLM What is claimed is:

1. A method for obtaining an immunostimulating reconstituted influenza virosome (IRIV) comprising: a. isolating a mixture including influenza viral membrane phospholipids and a trimeric influenza hemagglutinin protein (HA) or a peptide with the amino terminal 21 amino, acid residue segment of the HA_2 using octaethyleneglycol mono (n-dodecyl) ether (OEG) detergent; b. dispersing a glycerophospholipid and **cholesterol** in an aqueous solution so as to obtain an **unilamellar liposome**; c. combining the mixture and the **liposome** so as to obtain an **unilamellar** immunostimulating reconstituted influenza virosome (IRIV) and; d. adsorbing or attaching onto the surface of the IRIV a combination of immunostimulating pathogenic antigens; wherein the HA retains biological fusion activity equivalent to native HA at a pH value of about 5.0.

2. The method of claim 1 wherein the glycerophospholipid is **phosphatidylcholine** or phosphatidylethanolamine.

3. The method of claim 1 wherein the glycerophospholipid is a mixture of **phosphatidylcholine** and phosphatidylethanolamine in a ratio of about 1:1 to about 20:1.

4. The method of claim 3 wherein the ratio is about 10:1.

5. An IRIV made by the method of claim 1 wherein the trimeric influenza hemagglutinin protein (HA) or the peptide with the amino terminal 21 amino acid residue segment of the HA_2 has biological fusion activity equivalent to native HA at a pH value of about 5.0, and having immunostimulatory effect against the antigens.

6. An IRIV of claim 5 wherein the antigens are selected from the group consisting of viruses, parasites and bacteria.

7. An IRIV of claim 5 wherein the viruses are selected from the group consisting of Hepatitis A, B, C, D or E, polio virus, **HIV**, rabies virus, influenza virus, and parainfluenza virus.

8. An IRIV of claim 5 wherein the parasite is Plasmodium falciparum.

9. An IRIV of claim 5 wherein the bacteria is Clostridium tetani.

10. A method for obtaining an immunostimulating reconstituted influenza virosome (IRIV) comprising: a. isolating a mixture including influenza viral membrane phospholipids and a trimeric influenza hemagglutinin protein (HA) or a peptide with the amino terminal 21 amino acid residue segment of the HA_2 using octaethyleneglycol mono (n-dodecyl) ether (OEG) detergent; b. dispersing a glycerophospholipid and **cholesterol** in an aqueous solution so as to obtain an **unilamellar liposome**; c.

combining the mixture and the **liposome** so as to obtain an **unilamellar** IRIV having an essentially reconstituted viral envelope; and d. adsorbing or attaching onto the surface of the IRIV an immunostimulating pathogenic antigen other than a Hepatitis A antigen.

11. The method of claim 10 wherein the HA retains biological fusion activity equivalent to native HA at a pH value of about 5.0.

12. The method of claim 10 wherein the glycerophospholipid is **phosphatidylcholine** or phosphatidylethanolanine.

13. The method of claim 10 wherein the glycerophospholipid is a mixture of **phosphatidylcholine** and phosphatidylethanolamine in a ratio of about 1:1 to about 20:1.

14. The method of claim 13 wherein the ratio is about 10:1.

15. An IRIV made by the method of claim 11 wherein the trimeric influenza hemagglutinin protein (HA) or the peptide with the amino terminal 21 amino acid residue segment of the HA_2 retain biological fusion activity equivalent to native HA at a pH value of about 5.0, and having an immunostimulatory effect against the antigen.

16. An IRIV of claim 15 wherein the antigen is selected from the group consisting of viruses, parasites and bacteria.

17. An IRIV of claim 15 wherein the virus is selected from the group consisting of Hepatitis B, C, D or E, polio virus, **HIV**, rabies virus, influenza virus, and parainfluenza virus.

18. An IRIV of claim 15 wherein the parasite is Plasmodium falciparum.

19. An IRIV of claim 15 wherein the virus is **HIV**.

20. An IRIV of claim 15 wherein the bacteria is Clostridium tetani.

TI Immunostimulating and immunopotentiating reconstituted influenza virosomes and **vaccines** containing them

AI US 1994-225740 19940411 (8) <--

SUMM The invention relates to immunostimulating and immunopotentiating reconstituted influenza virosomes (IRIVs) and to **vaccines** containing them.

SUMM . . . including the self aggregation characteristic of antigens, such as the soluble antigen of hepatitis B virus. In the case of **liposomes** or oily droplets, there is a combined effect of particulateness and slow absorption, such as with alum precipitation.

SUMM . . . suppressor pathways in the immune responses. Particularly if the intravenous route is used (see Nossal, G. J. V., New Generation **Vaccines**, Marcel Dekker, Inc., New York, Basle, eds. Woodrow, Levine, 85, 1990). Slow release from a subcutaneous depot site permits extensive. . . Slow release is favored by adsorbing antigens onto aluminum hydroxide ("alum precipitation"); placing antigens into water-in-oil emulsions; incorporating antigens into **liposomes**; and other similar manipulations. This method is conceptually close to the one described in section A.

SUMM If a particular **vaccine** is highly immunogenic, the adjuvant effect of this **vaccine**, and also the characteristics it may possess for guiding the response toward a particular immunological pathway, may "spill over" into. . . Pure proteins co-administered with the parasite extracts will also evoke an IgE response (see Nossal, G. J. V., New Generation **Vaccines**, Marcel Dekker, Inc., New York, Basle, eds. Woodrow, Levine, 85, 1990). Presumably, this effect is somehow connected to the production. . .

SUMM . . . 1982), and Smith, G. L., Macket, M. and Moss, B. (Nature, 302:490, 1983), who genetically engineered the genome of the **vaccinia** virus to additionally include genes coding for important host-protective

antigens of various pathogens. These are synthesized by the infected cell together with **vaccinia** virus particles and antigens. An improvement of this concept was introduced by Langford, C. J. , et al. (Mol Cell.. . . gene encoding the soluble S antigen of Plasmodium falciparum, and inserted the resulting hybrid gene into the genome of a **vaccinia** virus. The construct caused a significantly enhanced immunogenicity. Live Salmonella, BCG and measles virus have also been successfully used for the expression of foreign antigen. Thus, the advantages of a live attenuated **vaccine** can be combined with those of a **vaccine** based on viruses containing recombinant DNA.

SUMM A further development of this idea is to insert genes for various interleukins into genetically engineered **vaccinia** viruses already carrying genes for important antigens. For example, the immune response to **vaccinia** virus itself can be markedly enhanced by the insertion of the IL-2 gene into the viral genome, permitting immunodeficient mice. . .

SUMM . . . such as saponin or Quil A.backslash. in immunostimulating complexes (iscoms) have been used in a number of experimental and veterinary **vaccines**. They improved the immunogenicity of several antigens, especially of viral membrane proteins.

SUMM . . . proinflammatory and encephalopathogenic potential. Surface active agents display a number of side reactions: they are irritating, proinflammatory, they bind to **cholesterol** and lyse cells. Interleukins can provoke systemic reactions and, therefore, routine use in mass **vaccination** may be undesirable.

SUMM . . . the antigen particulate often goes in parallel with a significant loss of the amount of antigen. The immunostimulatory effect of **liposome**-associated antigen on the humoral response is a widely recognized phenomenon, but immunopotentiation is limited and the mechanism by which this. . .

DRWD FIG. 3 is a bar graph comparing IRIV-HAV **vaccines** versus Al-HAV **vaccines**.

DRWD FIG. 4 is a bar graph comparing the immunogenicity of IRIV-HAV **vaccines** with those of Al-HAV **vaccines**.

DRWD FIG. 6 is a schematic of the preparation of an IRIV-HAV-HBx combination **vaccine**.

DRWD FIG. 7 is a schematic of the preparation of an IVIR traveller **vaccine** having HAV, HBs, diphtheria and tetanus antigens attached to the surface.

DETD The solution to the above technical problem is achieved by providing the immunostimulating reconstituted influenza virosomes (IRIVs) and **vaccines** containing said IRIVs which are characterized in the claims. These IRIVs can be used as vehicles which actively transport desired. . .

DETD . . . phospholipids or a mixture thereof. At least it contains two different compounds selected from the group of glycero-phospholipids, such as **phosphatidylcholine** or phosphatidylethanolamine, and **cholesterol**. **Phosphatidylcholine** and phosphatidylethanolamine are preferred, in particular in a ratio of 4:1. In preferred embodiments of the present invention, the ratio. . .

DETD . . . influenza virus envelope's membrane part. In a preferred embodiment the essentially reconstituted functional virus envelopes exhibit the form of a **unilamellar** bilayer. An example of such a lacking component is the matrix protein of the natural influenza virus envelope.

DETD . . . as a "recognition antigen" since most humans can be considered "primed" to HA due to prior exposure through disease or **vaccination**.

DETD . . . all human beings have antibodies against influenza antigen. These antibodies originate either from a previous influenza infection or from a **vaccination**) speeds entry of antigens recognized by said antibodies not only into macrophages, but also into lymphoid follicles, in which antigens. . .

DETD . . . by which said antigen is transported to lymphoid follicles and elsewhere in lymphatic tissue (Nossal, G. J. V., New Generation **Vaccines**, ed. Woodrow, G. C. and Levine, M. M., Marcel Dekker, Inc., 85, 1990). The mechanism would be an adjunct to. . .

DETD . . . bacteria or parasites, or a toxin. Examples of viruses are hepatitis A, B, C, D or E virus, Polio virus, **HIV**, Rabies virus, Influenza virus or Parainfluenza virus. Examples of bacteria are Pseudomonas, Kiebsiella, E. coli Salmonella typhi, Haemophilus influenzae, Bordetelia. . .

DETD In another embodiment, the present invention relates to a **vaccines** containing an IRIV of the present invention. The **vaccines** can contain IRIVs having a combination of different antigens on the surface of each IRIV, such as the combination IRIV, or the **vaccine** can contain a plurality of different types of IRIVs, each having a different antigen displayed on its surface so as to make a "cocktail" **vaccine**. Optionally, these **vaccines** additionally contain a suitable pharmaceutically acceptable carrier and/or diluent. These **vaccines** can be administered in conventional routes and dosages.

DETD (A) A dispersion of **phosphatidylcholine** (e.g., lecithin, SIGMA) (75%), phosphatidylethanolamine (SIGMA) (20%) and **cholesterol** (SIGMA) (5%) (all phospholipids 1-2% (w/v)=0.013-0.027M) in 0.1M NaCl containing 0.01M Tris/HCl, pH 7.3 was prepared by mixing these compounds. . .

DETD . . . ultrasonification apparatus (Bransonic, Branson Europe BV, frequency 50 kHz ±10%). 10 seconds of ultrasonic shocks repeated every minute yielded small **unilamellar** IRIVs. The sample volumes and column dimensions were such that a complete separation of IRIVs eluted at the void volume. . .

DETD **Phosphatidylcholine** (**PC**; Sigma Chemical Co., St. Louis, Mo.) and phosphatidylethanolamine (PE; Sigma) (75%:25% wt/wt) were suspended in 0.01M Tris - 0.1M NaCl,. . . fractions, which contained the IRIV, were pooled and re-chromatographed under identical conditions. The IRIV possessed an average diameter of approximately **150 nm**.

DETD Production of an HAV-IRIV **Vaccine**

DETD . . . A preservative (thiomersal) was added to a final dilution of 10_{-4}. Aliquots of 0.6 ml of the final bulk **vaccine** were filled into **vaccine** vials under sterile conditions. Safety and potency tests were performed according to international regulations.

DETD Preparation of an Anti-idiotype IRIV **Vaccine** Against Hepatitis C

DETD . . . Paris, 125 C:373, 1974]). The major advantage of using anti-id antibodies (Ab2) for eliciting antigen-specific antibodies (Ab3) is that the **vaccine** recipient is never in contact with infectious agents or materials containing foreign genes.

DETD The anti-idiotype IRIV **vaccine** against hepatitis C was prepared as follows: Sheep were immunized with an Ab1 (dissolved of a concentration of 1 mg/ml. . .

DETD . . . was diluted to a protein concentration of 50 μg/ml with PBS, pH 7.4, and portioned in 0.6 ml aliquots in **vaccine** vials.

DETD Safety and Immunogenicity of Inactivated Hepatitis A **Vaccines**: Comparison of IRIV-HAV Prepared According to Example 1 with Alum-absorbed **Vaccine**

DETD . . . number ATCC CCL 171). The virus was inactivated by treatment with formaldehyde (0.05%) at 37° C. for 10 days. Two **vaccine** series were tested. **Vaccine** series 1 consisted of inactivated virus linked to IRIVs according to Example 1 (A) (IRIV-HAV). **Vaccine** of series 2 was an alum-absorbed preparation containing 0.4% $Al(OH)_3$ (Al-HAV).

DETD Both **vaccines** contained 150 ng of HAV antigen per 0.5 ml dose. Seronegative adult volunteers (two groups of 15 persons each) received. . . chemistry were detected. With respect to local reactions, IRIV preparations provoked a significantly lower percentage of reactions than the alum-absorbed **vaccine**. The results of these experiments are summarized in FIG. 3.

DETD . . . the range of the anti-HAV antibody titer. Thus, the range of the anti-HAV antibody titers for the IRIV and alum-adsorbed **vaccine** formulations on day 21 was 82-988 and 69-844, respectively. The geometric mean titer (GMT) (range) for the IRIV and alum-adsorbed **vaccine** formulations on day 28 was 453 mlU/ml (92-1210) and 361 mlU/ml (60-929), respectively. Thus, the IRIV preparations of the present invention are superior to alum-adsorbed **vaccines**.

DETD . . . a phase I clinical study with 120 human volunteers it could be

demonstrated that one single IRIV adjuvanted hepatitis A **vaccine** dose induced protective antibody titers against hepatitis A which were 7 times higher than the antibody titer after the alum. . .

DETD A total of 120 HAV seronegative (<10 mlU/ml) healthy adults were randomized to receive either fluid, alum-adsorbed, or IRIV **vaccine** according to Example 1 (B). The **vaccine** (0.5 ml) was administered intramuscularly into the deltoid region. Volunteers were observed for approximately 30 minutes after **vaccination** for immediate-type reactions. Each volunteer was asked to record all adverse reactions on a report sheet for the 4 days. . .

DETD Each **vaccine** formulation contained 1 μg of HAV antigen per 0.5 ml dose. One dose of the IRIV-HAV formulation also contained 10 μg of influenza HA and 125 μg total phospholipids. All three **vaccines** were found to be sterile and nontoxic for animals by standard test methods. In addition, all 3 formulations elicited a. . .

DETD . . . immunization are shown in Table I. Pain at the injection site was the most frequently reported complaint with all the **vaccines**. Such discomfort was classified as moderate by one **vaccinee** (2.5%) who received the fluid formulation, 9 (23%) who were immunized with the alum-adsorbed **vaccine**, and one (2.5%) who received the IRIV preparation. Severe pain was reported by one subject who received the alum-adsorbed **vaccine**. All other subjects who reported a "painful" reaction graded it as mild. Immunization with the alum-adsorbed **vaccine** was associated with a significantly ($P<0.01$) higher incidence of both pain and swelling/induration as compared to either the fluid or IRIV formulations. No systemic reactions attributable to **vaccination** were noted.

DETD The anti-HAV antibody response engendered 14 days after **vaccination** is shown in Table II. Immunization with the fluid **vaccine** yielded a geometric mean titer (GMT) of 15.7 mlU/ml with 30% of subjects seroconverting (>20 mlU/ml). While the alum-adsorbed **vaccine** induced both a modestly higher GMT (21.3 mlU/ml) and seroconversion rate (44%), neither was significantly greater than that obtained with the fluid **vaccine**. In contrast, the IRIV **vaccine** formulation elicited a far more vigorous antibody response. The GMT of 139.8 was significantly ($P<0.0001$) higher compared to either of the other two **vaccines**. All but one **vaccinee** possessed >100 mlU/ml. Of greater importance was the fact that all **vaccinees** seroconverted by day 14 compared to less than 50% for the other **vaccine** formulations ($P<0.005$).

DETD TABLE I

Adverse Reactions Associated with Immunization

Vaccine	Local reactions (%) Pain	Swelling/ Induration Redness	Systemic reactions (%) Fever	Headache	Malaise
Fluid	42*	0.parallel.	0	0	0
$Al(OH)_3$ -	88+	23.paragraph.	0	0	0
adsorbed IRIV	25.sctn.				

. . .

DETD TABLE II

Immunogenicity of Fluid, $Al(OH)_3$ -Adsorbed, and IRIV-Adjuvanted Hepatitis A **Vaccines**

Vaccine	Geometric mean titer (range) in mIU/ml	Seroconversion rate

Formulation

	Day 0	Day 14	mIU/total (%)
Fluid	<10	15.7 (<10-100)*	12/40 (30%).parallel.
$Al(OH)_3$ -adsorbed	<10	21.3 (<10-100)+	18/40 (44%).paragraph.
IRIV-adjuvanted	<10	139.8 (25-300).sctn.	40/40 (100%)**

Subjects received a single dose of **vaccine** on day 0.
.sctn. vs * or +: P < 0.0001
** vs .parallel. or .paragraph.: P < 0.005

DETD . . . IRIVs contained 500 RIA Units of HAV and 10 μg of yHBs. The other group was immunized with two separate **vaccines**, one the hepatitis.

DETD A **vaccine** of Example 3 above containing 500 RIA units of HAV per dose, and the other a conventional alum adsorbed yHBs **vaccine** containing 0.25 mg per dose of yHBs.

DETD The results in Table III show that the IRIV-yHBs **vaccine** induced a higher GMT than the conventional Alum-yHBs **vaccine**. The antibody titers against HAV were comparable in both groups. Furthermore, the clinical acceptability of the IRIV **vaccine** was significantly better than that of the conventional **vaccine**: 67% of the volunteers **vaccinated** with the alum-based **vaccine** reported adverse reactions at the site of injection as compared to only 9% of the volunteers immunized with the IRIV **vaccine**.

DETD TABLE III

COMPARISON OF THE IMMUNOGENICITY OF IRIV ADJUVANTED yHBs ANTIGEN WITH A COMMERCIAL ALUM ADJUVANTED **VACCINE**

Effect of Booster

Vaccine	A B G.M.T. IU/ml Day 0	A B G.M.T. IU/ml Day 28	Efficiency Factor Mean of each single case
IRIV-HBs	343	13204	107
Alum-HBs	292	6373	30

Each group consisted of 6 volunteers.
One **vaccine** dose contained 10 μg of yHBs antigen.

DETD . . . simultaneously coupled to the surface of IRIVs as described in Example 3 (see FIG. 7). These IRIVs were used to **vaccinate** 15 volunteers. For comparison, a second group of 23 volunteers were **vaccinated** first with IRIV-HAV (500 RIA Units), and then separately with standard diphtheria/tetanus **vaccine** (4 and 40 IU, respectively) adsorbed onto alum-HGsAG (20 μg) on alum basis (Alumdite).

DETD . . . were comparable in both groups. Antibodies against yHBs were not tested in this study. Again, the tolerance to the IRIV-based **vaccines** was significantly better than that to the alum-based **vaccines** as shown in Table V. The IRIV supercombination, therefore, can be used to immunize with administration of a single dose. . .

DETD TABLE V

COMARISON OF SIDE REACTIONS COMBINED COMMERCIAL DI, TE, HEPATITIS A AND HEPATITIS B **VACCINES** VERSUS AN IIV SUPERCOMBI WITH THE SAME ANTIGEN

Side Reactions

	Commercial **Vaccines**	IRIV Supercombi

Pain grade 2 or 3
82% 24%
Induration 41% 23%
Redness 37% 9%
Redness average area left: 3800 mm^2
2 mm^2
right: 1034. . .

DETD . . . compared with live influenza virus in fusion activity with model membranes. FIG. 8 shows the kinetics of fluorescence de-quenching with DOPC-**cholesterol liposomes**. The increase in fluorescence is expressed in % florescence de-quenching (FDQ), calculated according to Luscher and Gluck.

. . . 21 amino, acid residue segment of the HA_2 using octaethyleneglycol mono (n-dodecyl) ether (OEG) detergent; b. dispersing a glycerophospholipid and **cholesterol** in an aqueous solution so as to obtain an **unilamellar liposome**; c. combining the mixture and the **liposome** so as to obtain an **unilamellar** immunostimulating reconstituted influenza virosome (IRIV) and; d. adsorbing or attaching onto the surface of the IRIV a combination of immunostimulating. . .
2. The method of claim 1 wherein the glycerophospholipid is **phosphatidylcholine** or phosphatidylethanolamine.

3. The method of claim 1 wherein the glycerophospholipid is a mixture of **phosphatidylcholine** and phosphatidylethanolamine in a ratio of about 1:1 to about 20:1.

. . . 5 wherein the viruses are selected from the group consisting of Hepatitis A, B, C, D or E, polio virus, **HIV**, rabies virus, influenza virus, and parainfluenza virus.

. . . 21 amino acid residue segment of the HA_2 using octaethyleneglycol mono (n-dodecyl) ether (OEG) detergent; b. dispersing a glycerophospholipid and **cholesterol** in an aqueous solution so as to obtain an **unilamellar liposome**; c. combining the mixture and the **liposome** so as to obtain an **unilamellar** IRIV having an essentially reconstituted viral envelope; and d. adsorbing or attaching onto the surface of the IRIV an immunostimulating. . .
12. The method of claim 10 wherein the glycerophospholipid is **phosphatidylcholine** or phosphatidylethanolanine.

13. The method of claim 10 wherein the glycerophospholipid is a mixture of **phosphatidylcholine** and phosphatidylethanolamine in a ratio of about 1:1 to about 20:1.

. . . claim 15 wherein the virus is selected from the group consisting of Hepatitis B, C, D or E, polio virus, **HIV**, rabies virus, influenza virus, and parainfluenza virus.

19. An IRIV of claim 15 wherein the virus is **HIV**.

L27 ANSWER 5 OF 8 USPATFULL on STN
1998:14497 Solid fat nanoemulsions as **vaccine** delivery vehicles.
Anselem, Shimon, Rehovot, Israel
Lowell, George H., Baltimore, MD, United States
Aviv, Haim, Rehovot, Israel
Friedman, Doron, Carmei Yosef, Israel
Pharmos Corporation, New York, NY, United States (U.S. corporation)The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 5716637 19980210
WO 9426255 19941124
APPLICATION: US 1995-553350 19951116 (8) <--
WO 1994-US5589 19940518 19951116 PCT 371 date 19951116 PCT 102(e) date

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention provides pharmaceutical **vaccine** compositions that are nanoemulsions of particles having a lipid core which is in a solid or liquid crystalline phase at 25° C., and which is surrounded by at least one phospholipid bilayer for the parenteral, oral, intranasal, rectal, vaginal or topical delivery of both hydrophilic and lipophilic immunogens. The particles have a mean diameter in the range of 10 to 250 nm and the immunogen is incorporated therein, either intrinsically prior to the homogenization process or extrinsically thereafter.

CLM What is claimed is:

1. A pharmaceutical composition for the administration of antigen which comprises a nanoemulsion of a plurality of noncellular lipid particles having a mean diameter of about 10 to 250 nm, as determined on a weight basis, the particles being suspended in an aqueous continuous phase, wherein each said lipid particle comprises a lipid core composed of a lipid which is a solid or liquid crystal as determined in bulk at a temperature of about 25° C. or higher, and at least one phospholipid bilayer surrounding said core and encapsulating a portion of said aqueous continuous phase, said particles entrapping about 0.001 to 5% of an immunogen in said lipid core or in said encapsulated aqueous phase.

2. The pharmaceutical composition of claim 1 wherein the mean particle diameter of said lipid particles falls within the range of about 20 to 180 nm as determined on a weight basis.

3. The pharmaceutical composition of claim 2 wherein the particle diameter of at least 99% of said lipid particles falls within the range of about 50 to **150 nm** as determined on a weight basis.

4. The pharmaceutical composition of claim 2 wherein the lipid core comprises a fatty acid ester.

5. The pharmaceutical composition of claim 4 wherein the lipid core has a solid to fluid phase transition temperature below 37° C. as determined in bulk.

6. The pharmaceutical composition of claim 4 wherein the lipid core comprises a triglyceride.

7. The pharmaceutical composition of claim 6 wherein said triglyceride comprises a fatty acid moiety of C_{10} to C_{18}.

8. The pharmaceutical composition of claim 6 wherein said triglyceride is completely saturated.

9. The pharmaceutical composition of claim 6 wherein said triglyceride is selected from the group consisting of tricaprin, trilaurin, trimyristin, tripalmitin, and tristearin.

10. The pharmaceutical composition of claim 6 wherein the mole ratio of phospholipid to total lipid is in the range of from 0.1:1 to 0.5:1.

11. The pharmaceutical composition of claim 6 wherein the weight ratio of phospholipid to triglyceride is in the range of from 0.5:1 to 1.5:1.

12. The pharmaceutical composition of claim 4 wherein said phospholipid is a **phosphatidylcholine**.

13. The pharmaceutical composition of claim 12 wherein said **phosphatidylcholine** is egg **phosphatidylcholine**.

14. The pharmaceutical composition of claim 12 wherein said **phosphatidylcholine** has a transition temperature below 25° C.

15. The pharmaceutical composition of claim 12 wherein said **phosphatidylcholine** is saturated.

16. The pharmaceutical composition of claim 1 wherein said lipid particle contains **cholesterol** or cholesteryl esters.

17. The composition of claim 1 wherein the immunogen is hydrophilic, lipophilic, or amphophilic.

18. The composition of claim 1 wherein the immunogen is a peptide, protein, or glycoprotein.

19. The composition of claim 18 wherein the antigen is the gp160 envelope protein of the **HIV** virus, or a fragment thereof.

20. The composition of claim 18 wherein the antigen is the surface glycoprotein of a Leishmania parasite, or a fragment thereof.

21. The composition of claim 20 wherein the surface glycoprotein or peptide is covalently conjugated to a hydrophobic component.

22. The composition of claim 21 wherein the hydrophobic component is lauryl-cysteine.

23. The composition of claim 1 wherein the immunogen is a protein toxoid.

24. The composition of claim 23 wherein the immunogen is Staphylococcus Enterotoxin B toxoid.

25. The composition of claim 1 wherein the immunogen is complexed with a proteosome.

26. The composition of claim 1 wherein the nanoemulsion further comprises a bioadhesive or mucoadhesive macromolecule.

27. The composition of claim 26 wherein the said mucoadhesive macromolecule is a polymer.

28. The composition of claim 26 wherein the said mucoadhesive macromolecule is selected from the group of acidic nonnatural polymers consisting of polymers and copolymers containing acrylic acid units, polymers and copolymers containing methacrylic acid units, and poly(methylvinylether/maleic anhydride) copolymer.

29. The composition of claim 28 wherein the said polymer is polyacrylic acid.

30. The composition of claim 1 which contains no added muramyl peptides.

31. The pharmaceutical composition of claim 1 wherein said lipid particle is substantially free of lipase and phospholipase activity.

32. A method for delivery of an immunogen to an animal, comprising administering to said animal a pharmaceutical according to claim 1.

33. The method of claim 32 wherein the mean diameter of the lipid particles in said composition is in the range of about 20 to 180 nm.

34. The method of claim 32 wherein said composition is administered parenterally, orally, intranasally, or topically, thereby providing enhanced immunogenicity.

35. The method of claim 32 wherein said composition is administered to mucosal surfaces, thereby achieving mucosal immunity.

36. A method for making a nanoemulsion for administration of an immunogen, comprising the steps of: preparing a mixture comprising phospholipid and triglyceride in the weight ratio range of about 0.5:1 to 1.5:1 wherein said triglyceride has a solid to liquid phase transition temperature of greater than 25° C.; suspending said mixture in an aqueous solution at a temperature below the solid to liquid transition temperature of the triglyceride; reducing the size of the suspension to yield a nanoemulsion of lipid particles having a mean particle diameter of between about 10 nm and 250 nm; and incorporating an immunogen in the nanoemulsion.

37. The method according to claim 36 for preparing the composition of the nanoemulsion by an intrinsic procedure, where the immunogen is added before homogenization of water and oil phases.

38. The method of claim 36 for preparing the composition of the nanoemulsion by an extrinsic procedure, where the immunogen is added externally by mixing with the previously prepared plain nanoemulsion.

39. A pharmaceutical composition comprising dehydrated lipid particles containing an antigen for administration as a nanoemulsion, wherein said lipid particles comprise a lipid core surrounded by at least one phospholipid layer, said lipid core is composed of lipid in a solid or liquid crystalline phase at least about 25° C. as determined in bulk, and said lipid particles have a mean diameter upon rehydration of about 10 to 250 nm.

40. The pharmaceutical composition of claim 39 further comprising a cryoprotectant.

41. The pharmaceutical composition of claim 40 wherein said cryoprotectant is selected from the group consisting of glucose, sucrose, lactose, maltose, trehalose, dextran, dextrin, cyclodextrin, polyvinylpyrrolidone, and amino acids.

42. The pharmaceutical composition of claim 40 wherein said cryoprotectant is present in a range of from 0.1% to 10% by weight compared to lipid.

43. The pharmaceutical composition of claim 39 wherein said lipid particles contain an immunogen.

44. A method for delivering an antigen to an animal comprising administering to said animal a pharmaceutical composition according to claim 39.

TI Solid fat nanoemulsions as **vaccine** delivery vehicles

AI US 1995-553350 19951116 (8) <--
WO 1994-US5589 19940518
19951116 PCT 371 date
19951116 PCT 102(e) date

AB The present invention provides pharmaceutical **vaccine** compositions that are nanoemulsions of particles having a lipid core which is in a solid or liquid crystalline phase at. . .

SUMM The present invention concerns methods and compositions for delivery of **vaccines** by parenteral and other routes of administration. More particularly, it concerns stable lipid-in-water nanoemulsions or emulsomes containing small lipid particles. . .

SUMM In the past, the risks of whole-pathogen **vaccines** and limited supplies of useful antigens posed barriers to development of practical **vaccines**. Today, the tremendous advances of genetic engineering and the ability to obtain many synthetic recombinant protein antigens derived from parasites, viruses, and bacteria has revolutionized the development of new generation **vaccines**.

SUMM Several reports describing the improvement of immune response achieved

by the association of antigens with lipid carriers such as **liposomes** or microparticles like polymeric biodegradable microcapsules have been published (C. R. Alving, **Liposomes** as Carriers of **Vaccines**, in "**Liposomes**: From Biophysics to Therapeutics", M. J. Ostro, ed., Ch. 6, Marcel Dekker Inc., New York, 1987, pp. 195-218; J. H.. . .

SUMM . . . carriers by encapsulation or entrapment, or embedded in their surface, they show enhanced ability to evoke a strong immune response. **Vaccines** formulated in lipid carriers probably enhance antibody production by increasing activity along both pathways of stimulation of immune system described. . .

SUMM Most **vaccine** adjuvants are also surface-active, or have a special surface interface. Surface-active agents concentrate at the surface formed by the interface. . .

SUMM The use of **liposomes** as drug delivery systems has been known for some time, and comprehensive review articles on their properties and clinical applications are available; see, e.g., Barenholz and Amselem, in "**Liposome** Technology", 2nd ed., G. Gregoriadis, ed., CRC Press, 1992; Lichtenberg and Barenholz, in Methods for Biochemical Analysis, 33, D. Glick, ed., 1988. A **liposome** is defined as a structure consisting of one or more concentric lipid bilayers separated by water or aqueous buffer compartments.. . . from 20 nm to 10 μm. They are classified according to their final size and preparation method as: SUV, small **unilamellar** vesicles (0.5-50 nm); LUV, large **unilamellar** vesicles (100 nm); REV, reverse phase evaporation vesicles (0.5 μm); and MLV, large multilamellar vesicles (2-10 μm).

SUMM An extensive literature exists on immunologic characteristics of **liposomes**, and numerous reviews on their potential as **vaccine** carriers have been published especially by C. R. Alving and co-workers who developed the first injectable liposomal **vaccine** for human use administered to 30 volunteers in a Phase I study (Fries et al, Proc. Natl. Acad. Sci. USA,. . .

SUMM . . . vehicle which is denoted herein as a solid fat nanoemulsion or "emulsome." These compositions have features which are intermediate between **liposomes** and oil-in-water emulsions. Emulsome particles contain a hydrophobic core, as in standard oil-in-water emulsions, but surrounded and stabilized by one or more bilayers or envelopes of phospholipid molecules, as in **liposomes** (FIGS. 1A, 1B and 1C).

SUMM Emulsomes, having the characteristics of both **liposomes** and emulsions, provide the advantages of high loading of hydrophobic bioactive compounds in the internal solid lipid core and the. . .

SUMM The emulsome technology represents a new type of lipid-based encapsulation technology that has potential usefulness as carriers of **vaccines** and adjuvants enhancing the immunogenicity of antigens incorporated intrinsically or extrinsically into the particles.

SUMM Emulsome-**vaccine** formulations containing antigens can provide the surface interphase necessary for proper orientation of the adjuvant active material, resulting in enhanced. . .

SUMM . . . antigen to a surface or presentation of a special type of surface for antigen adsorption, as in the case of emulsome-**vaccines**, appears to be critical for much of the biological activity of most agents reported as adjuvants. Therefore the emulsome technology can serve as effective vehicles or delivery systems for human and veterinary **vaccines**.

SUMM The use of emulsomes as a **vaccine** delivery system has other demonstrable advantages. Emulsomes of this invention provide effective pharmaceutical delivery for a broad variety of both. . .

SUMM Additional advantages of the emulsome technology for delivery of **vaccines** are: antigens and adjuvants can be incorporated simultaneously in the same formulation due to the hydrophilic-hydrophobic nature of the emulsome. . . be obtained; emulsomes can be formulated to act as depot for slow release of antigens avoiding the need for repeated **vaccinations**; the manufacturing technique of emulsomes is relatively simple and easy to scale-up.

SUMM The emulsome-**vaccine** formulations of this invention do not include any polyoxypropylene-polyoxyethylene block polymer, trehalose dimycolate, cell wall skeleton, or any immunostimulatory mycobacteria. . .

SUMM ... may be varied to suit the particular antigen used. Moreover, such formulations may enhance both humoral and cell-mediated immunity (CMI). . . . or muramyl-peptide derivatives they have, unlike Freund's adjuvants, great safety potential. They may be especially applicable to antigens that are **vaccine** candidates to protect against biologic toxins or infectious agents which have natural hydrophobic moieties as a component including transmembrane viral,. . .

SUMM In addition to parenteral **vaccination**, another aspect of this invention is to provide emulsome compositions and methods to achieve mucosal immunity by using emulsome preparations. . .

DRWD FIGS. 1A, 1B and 1C are schematic illustrations of a **liposome**, an oil-in-water submicron emulsion droplet, and a proposed structure for an emulsome particle, respectively.

DRWD FIGS. 3A-C compares the ^{31} P-NMR spectra of submicron oil-in-water emulsion, emulsomes, and **liposomes**, recorded in the absence ("A" series) and presence ("B" series) of $PrCl_3$ (30 mM).

DRWD . . . is a graph showing enhanced murine immunogenicity after parenteral immunization of mice with formalinized SEB-Toxoid antigen formulated in intrinsic emulsome **vaccine** compared to free antigen or alum-adjuvanted **vaccine**.

DRWD . . . 6 is a graph showing enhanced lapine immunogenicity of SEB-Toxoid F antigen after parenteral immunization of rabbits with extrinsic emulsome **vaccine** compared to free antigen.

DRWD FIGS. 7A and 7B are a pair of graphs showing protection of mice immunized with mucoadhesive emulsome **vaccines** containing SEB-Toxoid F antigen or SEB-Tox F complexed to proteosomes against intranasal challenge with SEB toxin in BALB/C mice D-galactosamine. . .

DRWD FIG. 8 is a graph showing increased immune response in rhesus monkeys immunized with anti-Leishmania intrinsic emulsome **vaccine** containing LC-467 lipopeptide antigen.

DETD This invention is directed to pharmaceutical compositions for the delivery of water-soluble and lipid-soluble **vaccines**, and to methods for preparing and using such compositions.

DETD . . . compounds which are soluble in hydrocarbon solvents and are of a structural type which includes fatty acids and their esters, **cholesterol** and cholesteryl esters, and phospholipids.

DETD . . . or minus the standard deviation falls within the range 20 to 180 nm, 40 to 160 nm, or 50 to **150 nm**. In other preparations, the mean and the standard deviation falls within the range 10 to 120 nm. In still more. . .

DETD . . . hydrogenated vegetable oils yield solid vegetable shortening (e.g., CRISCO), which may be used to prepare emulsomes which are free of **cholesterol** or cholesteryl esters.

DETD **Cholesterol** and cholesteryl esters optionally may be incorporated into the lipid core or the surrounding phospholipid envelope. **Cholesterol** and its esters change the packing structure of lipids, and in high concentrations they induce the formation of a liquid. . .

DETD Since **cholesterol** has a polar alcohol group, it tends to incorporate into the envelope monolayers or bilayers rather than into the lipid. . .

DETD . . . envelopes of emulsomes are natural phospholipids such as soybean lecithin, egg lecithin, phosphatidylglycerol, phosphatidylinositol, phosphatidyl-ethanolamine, phosphatidic acid, sphingomyelin, diphosphatidylglycerol, phosphatidylserine, **phosphatidyl-choline**, cardiolipin, etc.; synthetic phospholipids such as dimyristoylphosphatidylcholine, dimyristoyl-phosphatidylglycerol, distearoylphosphatidylglycerol, dipalmitoylphosphatidylcholine, etc.; and hydrogenated or partially hydrogenated lecithins and phospholipids.

DETD . . . preferably at least 75%, most preferably at least 90% on a molar basis. Examples of normal phase forming phospholipids are **phosphatidyl-choline** (lecithin), phosphatidylglycerol, and phosphatidylinositol. By contrast, phosphatidlyl-ethanolamine has a tendency to form reverse phases, with the polar head groups oriented. . .

DETD . . . a gel to liquid phase transition temperature greater above or below 25° C. Egg or soy phosphatidylcholines (egg or soy **PC**) are examples of phospholipids with transition temperatures well below room temperature. Dimyristoyl **phosphatidyl-choline** (DMPC) has a transition temperature slightly below room temperature.

DETD . . . human clinical studies; see, e.g., S. Amselem et al., J. Pharm. Sci. (1990) 79, 1045-1052; S. Amselem et al., J. **Liposome** Res. (1992) 2, 93-123. The significance of zeta potential in analyzing and predicting the properties of phospholipid bilayers is discussed in L. Sai-lung, Chapter 19, Vol. 1 in "**Liposome** Technology," 2nd ed., G. Gregoriadis, ed., CRC Press, Boca Raton, Fla. (1993), pp. 331-342. Both lipoidal particle size and particle stability vary as a function of zeta potential. For **liposomes**, zeta potential and particle size increase in proportion to the content of negatively charged phospholipid, up to 50 weight %. . .

DETD Emulsomes for use as **vaccine** vehicles contain an antigen of interest, usually an antigen bearing at least one epitope which is present on an organism which is a pathogen in the animal species to be **vaccinated**. In most cases, the antigen is a peptide, a protein, or a glycoprotein. However, other antigenic structures may be employed,. . .

DETD Since the emulsome particles provide a soluble or lipid-soluble immunogens can be incorporated in emulsome **vaccines** of the present invention. Examples of peptide antigens are: hydrophilic natural or synthetic peptides and proteins derived from bacteria, viruses and parasites, such as the recombinant gp160 envelope protein of the **HIV** virus; natural or synthetic glycoproteins derived from parasites, bacteria or viruses such as the native surface glycoprotein of Leishmania strain or subunit **vaccines** containing part of the glycopeptides alone or covalently conjugated to lipopeptides like lauryl-cystein hydrophobic foot; protein toxoids such as the. . .

DETD In making a **vaccine** with emulsome vehicle, the antigen of interest may be added to the organic solution of core lipid and phospholipid prior. . .

DETD Mucoadhesive Emulsome **Vaccines**

DETD Emulsome **vaccine** of the present invention optionally may contain a bioadhesive macromolecule or polymer in an amount sufficient to confer bioadhesive properties.. . .

DETD TABLE 1

	SME	Emulsome Dispersion of a solid **Liposome** Dispersion of phospho-lipids in water. . . usually absent	
Definition	Dispersion of an oil in water	fat or lipid in	usually
surfactant			
Co-surfactant	present	usually absent	usually absent
Lipophilic loading	up to 10 mg/ml	up to 100 mg/ml	up to 20 mg/ml
PC/total lipid (mol/mol)	0.01-0.1	0.1-0.5	0.6-1.0

DETD A further embodiment of the invention relates to methods for preparation of emulsome **vaccines** intrinsically and extrinsically as extensively detailed in the examples. In general, emulsome intrinsic formulations are prepared by emulsifying the antigen. . .

DETD . . . 10 nm and 250 nm, usually within the range 20 to 180 nm, and frequently within the range 50 to **150 nm**.

DETD . . . Holland) or EmulsiFlex.TM. homogenizer (Avestin Inc., Canada). High pressure Gaulin homogenization is described in detail in Brandl et al., in **Liposome** Technology, 2nd ed., G. Gregoriadis, ed., Vol. 1, Ch.

DETD . . . GH76-400 Extruder or Pressure Cell (Nucleopore, U.S.A.), rather than a high-shear homogenizer. The pressure extruder and the extrusion technique for **liposome** preparation are described in detail in S. Amselem et al., in **Liposome** Technology, 2nd ed., G. Gregoriadis, ed., Vol. 1, Ch. 28, CRC Press, Boca Raton, Fla., (1993), pp 501-525.

DETD To a 0.5 liter round-bottomed flask, 2.5 g of egg-lecithin, 2.5 g of tricaprin, 0.1 g of **cholesterol**, 0.1 g of oleic acid and 0.01 g of tocopherol succinate were added. The lipid mixture was dissolved in 50. . .

DETD To a 0.5 liter round-bottomed flask, 3.5 g of egg-lecithin, 3.5 g of tricaprin, 0.2 g of **cholesterol**, 0.2 g of oleic acid, and 0.05 g of tocopherol succinate were added. The lipid mixture was dissolved in 50. . .

DETD . . . (Coulter Electronics, England) working at the differential weight % operation mode of the instrument (Barenholz, Y., and Amselem, S. In "**Liposome** Technology", Gregoriadis G., ed., 2nd edition, Vol. 1, pp 527-616, CRC press, Florida, 1993).

DETD . . . the fraction of lipid exposed to the external medium. FIG. 3 shows phosphorous nuclear magnetic resonance (^{31} P-NMR) spectra of **liposomes**, emulsomes, and submicron oil-in-water emulsion recorded before and after the addition of the lanthanide ion Pr+3. The ^{31} P-NMR spectra. . .

DETD . . . aqueous medium) interacted with the Pr+3 ions demonstrating the existence of bilayer structures. The same picture was obtained for small **unilamellar liposomes** (FIG. 3B3) used as control, where the existence of phospholipid bilayer domains have been well documented (Barenholz, Y., and Amselem, S. In "**Liposome** Technology", Gregoriadis G., ed., 2nd edition, Vol. 1, pp. 527-616, CRC press, Florida, 1993).

DETD PREPARATION OF EXTRINSIC MUCOADHESIVE EMULSOME **VACCINE**

DETD To a round 0.5 liter round-bottomed flask, 1.75 gr of egg-lecithin, 1.75 gr of tricaprin, 70 mg of **cholesterol**, 70 mg of oleic acid, and 7 mg of tocopherol succinate were added. The lipid mixture was dissolved in 50. . . be added extrinsically and mixed with the emulsome carrier particles by gentle shaking in order to obtain the proper emulsome **vaccine**.

DETD PREPARATION OF INTRINSIC HEPATITIS B EMULSOME **VACCINE**

DETD To a round 0.25 liter round-bottomed flask, 2.5 gr of egg-lecithin, 2.5 gr of tricaprin, 100 mg of **cholesterol**, 100 mg of oleic acid, and 10 mg of tocopherol succinate were added. The lipid mixture was dissolved in 25. . . indicated the existence of a single homogeneous population of emulsomes with a mean particle diameter of 105+24 nm. The emulsome **vaccine** formulation was then 2-fold concentrated using a Filtron ultrafiltration stirred cell (Omega Series membrane with 10,000 molecular weight cutoff, Filtron. . .

DETD PREPARATION OF MUCOADHESIVE INTRINSIC ANTI-**HIV** ENVELOPE PROTEIN (qp160) EMULSOME **VACCINE**

DETD Antigen description and background: The urgency and high priority for developing an effective **vaccine** against the **human immunodeficiency virus** (**HIV**) are fully recognized. The reasons for using subunits of the virus as the basis of an **HIV vaccine** are the perceived overwhelming requirements for safety. Despite the high efficacy of many live attenuated viral **vaccines**, the requirement for product safety, especially in the case of retroviruses, has favored the subunit approach to the extent that. . . of the current candidate preparations in clinical prophylactic trials are of this type, being mainly gp160, the envelope protein of **HIV**, or part thereof. Studies have shown that gp160 attaches the virus to the cell and also facilitates the fusion of. . .

DETD To a round 0.25 liter round-bottomed flask, 0.24 gr of egg-lecithin, 0.24 gr of tricaprin, 20 mg of **cholesterol**, and 20 mg of oleic acid, and 2 mg of tocopherol succinate were added. The lipid mixture was dissolved in. . . indicated the existence of a single homogeneous population of emulsomes with a mean particle diameter of 158±57 nm. The emulsome **vaccine** formulation was then 5-fold concentrated using a

molecular weight cutoff, Filtron. . . gr of a 1% Carbopol solution was added and stirred for 15 min to confer mucoadhesive properties to the emulsome **vaccine** preparation. Glycerol (0.285 gr) were added thereafter to reach a physiological osmolarity. The pH was adjusted to 6.0 using a. . .

DETD PREPARATION OF MUCOADHESIVE INTRINSIC EMULSOME **VACCINE** CONTAINING ANTI-**HIV** ENVELOPE PROTEIN (gp160) COMPLEXED TO PROTEOSOMES

DETD . . . A. Wirtz, W. D. Zollinger and W. T. Hockmeyer. Science 240, 800-802, 1988; Lowell, G. H. 1990. In: "New Generation **Vaccines**". G. C. Woodrow and M. M. Levine (eds.), Marcel Dekker, Inc., New York, p. 141-160) and have been shown to be safe for human use in **vaccine** trials involving tens of thousands of humans in the development of anti-meningococcal **vaccines** (Zollinger, W. D. New and Improved **Vaccines** Against Meningococcal Disease. In: "New Generation **Vaccines**", G. C. Woodrow and M. M. Levine (eds.), Marcel Dekker, Inc., New York, p. 325-348). Furthermore, proteosomes confer mucosal immunogenicity upon non-immunogenic antigens when administered orally or intranasally. Such intranasal or oral proteosome **vaccines** induce up to 100% protection against lethal pneumonia or keratoconjunctivitis in experimental murine models of shigellosis (Orr, N., G. Robin,. . . R. Arnon and G. Lowell. 1993. Immunogenicity and efficacy of oral or intranasal Shigella flexneri 2a and Shigella sonnei proteosome-lipopolysaccharide **vaccines** in animal models. Infect. Immun. 61, 2390-2395).

DETD . . . and the mixture was gently shaken for 5 min. The subsequent steps involved in the preparation of the mucoadhesive emulsome **vaccine** were carried out as described in Example 6. The particle size volume % distribution of the resultant emulsome formulation showed. . .

DETD PREPARATION OF MUCOADHESIVE EXTRINSIC EMULSOME **VACCINE** CONTAINING gp160 ALONE OR COMPLEXED TO PROTEOSOMES

DETD PREPARATION OF INTRINSIC EMULSOME **VACCINE** CONTAINING STAPHYLOCOCCUS ENTEROTOXIN B TOXOID-F ANTIGEN

DETD . . . delivered via the respiratory route in lower animal species, non-human primates have been the primary animal model for development of **vaccines** to protect against respiratory challenge with SEB. Early work indicated that monkeys develop decreased sensitivity to repeated mucosal administration of. . . challenged with SEB toxin. These adverse reactions suggested that the formalinized SEB toxoid alone was not a candidate for parenteral **vaccine** development. Additionally, as the military threat would be by aerosolization, it was determined that studies on protection provided by serum. . .

DETD To a round 0.5 liter round-bottomed flask, 2.25 gr of egg-lecithin, 2.25 gr of tricaprin, 90 mg of **cholesterol**, 90 mg of oleic acid, and 9 mg of tocopherol succinate were added. The lipid mixture was dissolved in 50. . .

DETD PREPARATION OF EXTRINSIC EMULSOME **VACCINE** CONTAINING STAPHYLOCOCCUS ENTEROTOXIN B-TOXOID-F

DETD PREPARATION OF INTRINSIC EMULSOME **VACCINE** CONTAINING STAPHYLOCOCCUS ENTEROTOXIN B-TOXOID-F COMPLEXED TO PROTEOSOMES

DETD To a round 0.5 liter round-bottomed flask, 2.5 gr of egg-lecithin, 2.5 gr of tricaprin, 100 mg of **cholesterol**, and 100 mg of oleic acid, and 10 mg of tocopherol succinate were added. The lipid mixture was dissolved in. . .

DETD PREPARATION OF EXTRINSIC EMULSOME **VACCINE** CONTAINING STAPHYLOCOCCUS ENTEROTOXIN B-TOXOID-F COMPLEXED TO PROTEOSOMES

DETD PREPARATION OF INTRINSIC EMULSOME **VACCINE** CONTAINING LC-467 LEISHMANIA LIPOPEPTIDE ANTIGEN

DETD . . . Leishmania major gp63, has been cloned and sequenced. This protein, recombinantly expressed in live Salmonella, or given in a sub-unit **vaccine** as either the purified native gp63 or selected gp63 peptides (Jardim A., Alexander J., Teh S., Ou D, Olafson R.. . . extent of lesion development in murine models of cutaneous leishmaniasis when given with certain adjuvants. These results suggest that a **vaccine** to protect humans against leishmaniasis composed of defined purified components is a realistic goal. The sub-unit **vaccines** were

effective, however, only when administered with adjuvants containing Corynebacterium parrum (CPV) and poloxamer 407. Other adjuvants (Complete Freund's Adjuvant, . . .

DETD To a round 0.25 liter round-bottomed flask, 0.4 gr of egg-lecithin, 0.4 gr of tricaprin, 15 mg of **cholesterol**, and 15 mg of oleic acid, and 1.5 mg of tocopherol succinate were added. The lipid mixture was dissolved in. . . indicated the existence of a single homogeneous population of emulsomes with a mean particle diameter of 181±35 nm. The emulsome **vaccine** formulations were then 6.5-fold concentrated using a Filtron ultrafiltration stirred cell (Omega Series membrane with 10,000 molecular weight cutoff, Filtron. . .

DETD IMMUNOGENICITY OF EMULSOME **VACCINE** CONTAINING LC-467 LEISHMANIA LIPOPEPTIDE ANTIGEN

DETD The objective in the present example was to demonstrate immunogenicity and efficacy of LC-467 Leishmania lipopeptide emulsome **vaccine** to protect against severe morbidity of cutaneous leishmaniasis in murine models.

DETD . . . disease. The immunization protocol included two injections of the animals (8 mice/group) at weeks 0 and 3 with the experimental **vaccines** (50 μg peptide/mouse). At week 6 the mice were infected with live Leishmania parasites and the lesion size as function. . .

DETD TABLE 2

Vaccine Formulation	% Protection (reduction of lesion size compared to control mice)
LC-467 in saline	73
LC-467 in emulsomes	94

DETD ENHANCED MURINE IMMUNOGENICITY OF SEB TOX-F ANTIGEN AFTER PARENTERAL IMMUNIZATION WITH INTRINSIC EMULSOME **VACCINE** COMPARED TO FREE ANTIGEN OR ALUM-ADJUVANTED **VACCINE**

DETD . . . emulsome formulation was more effective in enhancing immunity to SEB antigens. The anti-SEB serum IgG titers obtained with the emulsome **vaccine** were higher than those obtained with the alum-adjuvanted formulation or free antigen.

DETD ENHANCED LAPINE IMMUNOGENICITY OF SEB TOX-F ANTIGEN AFTER PARENTERAL IMMUNIZATION WITH EXTRINSIC EMULSOME **VACCINE** COMPARED TO FREE ANTIGEN

DETD PROTECTION AGAINST SYSTEMIC CHALLENGE WITH SEB IN MICE IMMUNIZED PARENTERALLY WITH SEB TOXOID **VACCINES** FORMULATED WITH ALUM, EMULSOME, OR FREE ANTIGEN

DETD Mice immunized parenterally (Table 3) with Staphylococcus Enterotoxin B in mice immunized with SEB Toxoid-F **vaccine** formulated with emulsomes (as described in example 9) were significantly protected against systemic SEB challenge (100 ug toxin).

DETD . . . mice with protection against systemic challenge with 100 g of SEB. In the groups immunized with intrinsic emulsome-SEB Toxoid F **vaccine**, the survival was 100% while for animals immunized with free antigen or alum-adjuvanted **vaccine** the survival was only 20 to 40%.

DETD PROTECTION OF MICE IMMUNIZED WITH MUCOADHESIVE EMULSOME **VACCINES** CONTAINING SEB-TOXOID F ANTIGEN OR SEB-TOX F COMPLEXED TO PROTEOSOMES AGAINST INTRANASAL CHALLENGE WITH SEB TOXIN IN BALB/C MICE D-GALACTOSAMINE. . .

DETD BALB/C mice (10 animals/group) were immunized intranasally twice with 100 μg antigen doses of Mucoadhesive Emulsome **vaccines** containing either SEB-Tox F antigen or SEB-Tox F complexed to proteosomes. The mucoadhesive formulations were prepared as described in Example. . . Mice survival was determined 3 days post-challenge. FIGS. 7A and 7B clearly show that the mice immunized with the emulsome **vaccines** had the highest percent of survival either in the Tox F or Tox F complexed to proteosomes groups (70% and. . .

DETD INDUCTION OF MUCOSAL, INTESTINAL, AND SYSTEMIC IMMUNOGENICITY IN MICE

OR COMPLEXED TO PROTEOSOMES INCORPORATED IN EXTRINSIC MUCOADHESIVE EMULSOME **VACCINES**

DETD . . . different intervals. Murine sera, lung fluids, and intestinal fluids obtained after immunizations were analyzed by ELISA techniques using several specific **HIV** epitopes as the detecting antigens.

DETD Table 4 shows that emulsomes enhanced the mucosal immunogenicity of **HIV** envelope protein following intranasal immunization with **HIV** gp160 formulated with or without the proteosome **vaccine** delivery system. Specific systemic anti-**HIV** IgG antibody production was enhanced 8-fold in the sera of mice immunized with the antigen incorporated in emulsome **vaccine** formulation, and 128-fold in the sera of mice immunized with the gp160-proteosome-emulsome formulation compared to the free antigen. In addition,. . . intestinal and lung fluid IgA, respectively, compared to immunizing with gp160 alone. Table 4 also shows that intranasal immunization with **HIV** gp160 formulated with emulsomes enhances serum IgG **HIV** gp41 peptide epitope responses (C448, C41, and CKen) as measured by quantitative western blot analyses. In addition, formulation of the gp160-proteosome **vaccine** with emulsomes enhances the serum IgG **HIV** responses to each of seven gp120 and gp41 peptide epitopes tested with increased responses ranging from over 116,000 to 9,000,000.

DETD TABLE 4*

Vaccine Formulation				
Anti-gp160 IgG or IgA titers of sera, gavage, and lavage fluids as measured by ELISA:				
Anti-gp160	51,200	409,600	1,638,400	6,553,600
Serum IgG Anti-gp160	<50	<50. . .	9,025,282	
518) C41 (579-605)	210,167	1,833,409	1,189,986	2,469,425
CKen (735-752)	<100	73,621	15,119	2,697,628

*Bolded numbers indicate enhancement by emulsomes compared to same **vaccin** in saline without emulsomes. Underlined numbers indicate enhancement by proteosomes compared to same formulation without proteosomes.

DETD INCREASED IMMUNE RESPONSE IN RHESUS MONKEYS IMMUNIZED WITH ANTI-LEISHMANIA INTRINSIC EMULSOME **VACCINE** CONTAINING LC-467 LIPOPEPTIDE ANTIGEN

DETD The results in FIG. 8 show that the monkey group **vaccinated** with the emulsome **vaccine** resulted in the highest anti-Leishmania IgG antibody titer, whereas the control group and monkeys that received the LC-467 antigen alone. . .

DETD To a 0.5 liter round-bottomed flask, 3.5 g of egg-lecithin, 5.2 g of tricaprin, 0.14 g of **cholesterol**, 0.14 g of **cholesterol**, 0.14 g of oleic acid, and 0.05 g of tocopherol succinate were added. The lipid mixture was dissolved in 50. . .

DETD . . . polylactide ("Resomer L 104," MW=2,000 Da, Boehringer Ingelheim, Germany), 0.5 g of egg-lecithin, 0.5 g of tricaprin, 0.2 g of **cholesterol**, 0.2 g of oleic acid, and 0.02 g of tocopherol succinate were added. The lipid mixture was dissolved in 50. . .

. . . wherein the particle diameter of at least 99% of said lipid particles falls within the range of about 50 to **150 nm** as determined on a weight basis.

12. The pharmaceutical composition of claim 4 wherein said phospholipid

is a phosphatidylcholine.

13. The pharmaceutical composition of claim 12 wherein said **phosphatidylcholine** is egg **phosphatidylcholine**.

14. The pharmaceutical composition of claim 12 wherein said **phosphatidylcholine** has a transition temperature below 25° C.

15. The pharmaceutical composition of claim 12 wherein said **phosphatidylcholine** is saturated.

16. The pharmaceutical composition of claim 1 wherein said lipid particle contains **cholesterol** or cholesteryl esters.

19. The composition of claim 18 wherein the antigen is the gp160 envelope protein of the **HIV** virus, or a fragment thereof.

L27 ANSWER 6 OF 8 USPATFULL on STN
96:94336 Hepatitis A virus in a reconstituted influenza virosome and use as a **vaccine**.
Gluck, Reinhard, Spiegel/Bern, Switzerland
Mischler, Robert, Worblaufen, Switzerland
Schweiz. Serum- & Impfinstitut Bern, Bern, Switzerland (non-U.S. corporation)
US 5565203 19961015
APPLICATION: US 1993-965246 19930303 (7) <--
PRIORITY: EP 1991-107527 19910508
EP 1991-107647 19910510
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Disclosed are immunostimulating reconstituted influenza virosomes (IRIVs) comprising reconstituted functional virus envelopes containing an inactivated hepatitis A virus and an influenza hemagglutinin protein (HA) or a peptide with the amino terminal 21 amino acid residue segment of HA_2 which induces the fusion of the IRIVs with cellular membranes and the lysis of the IRIV. A **vaccine** containing the hepatitis A IRIVs is also disclosed.

CLM What is claimed is:

1. An immunostimulating reconstituted influenza virosome (IRIV) comprising the following components: (a) a mixture of phospholipids; (b) essentially reconstituted functional virus envelopes containing a trimeric influenza hemagglutinin protein (HA) or a peptide with the amino terminal 21 amino acid residue segment of HA_2 which has biological fusion activity equivalent to native HA at a pH value of about 5.0 wherein said biological fusion activity induces the fusion of said IRIV with cellular membranes and the lysis of said IRIV after endocytosis by antigen presenting cells; and (c) a whole, inactivated hepatitis A virus (HAV), said virus being attached or adsorbed to the surface of the IRIV; wherein the IRIV is substantially **unilamellar**.

2. The IRIV according to claim 1, wherein the essentially reconstituted virus envelopes contain a peptide with the amino terminal 21 amino acid residue segment of HA_2.

3. The IRIV according to claim 1, wherein said essentially reconstituted functional virus envelopes are in the form of a **unilamellar** bilayer.

4. The IRIV according to claim 1, wherein the HAV is hepatitis A virus strain RG-SB XA112 (CNCM I-1080).

5. The IRIV according to claim 1, wherein said mixture comprises **phosphatidylcholine** and phosphatidylethanolamine.

6. The IRIV according to claim 5, wherein **phosphatidylcholine** and phosphatidylethanolamine are in a weight ratio of 4:1.

7. The IRIV according to claim 1, wherein the weight ratio of a mixture of phospholipids:essentially reconstituted functional virus envelopes is from about 1:1 to about 20:1.

8. A **vaccine** containing an IRIV according to claim 1 optionally in combination with a suitable pharmacologically acceptable carrier and/or diluent.

9. A method of stimulating the immune system of a patient in need thereof comprising administering a suitable dosage for providing a protective immune response of an IRIV according to claim 1.

10. A method for the prophylaxis of hepatitis A infections comprising administering a suitable dosage for providing a protective immune response of an IRIV according to claim 1 to a patient in need thereof.

TI Hepatitis A virus in a reconstituted influenza virosome and use as a **vaccine**

AI US 1993-965246 19930303 (7) <--

AB . . . segment of HA_2 which induces the fusion of the IRIVs with cellular membranes and the lysis of the IRIV. A **vaccine** containing the hepatitis A IRIVs is also disclosed.

SUMM The invention relates to immunostimulating and immuno-potentiating reconstituted influenza virosomes (IRIVs) and to **vaccines** containing them.

SUMM . . . strategies, including the self-aggregation characteristic of antigens, such as the soluble antigen of hepatitis B virus. In the case of **liposomes** or oily droplets, there is a combined effect of particulateness and slow absorption, such as with alum precipitation.

SUMM . . . activating the suppressor pathways in the immune responses, particularly if the intravenous route is used; see Nossal, G.J.V., New Generation **Vaccines**, Marcel Dekker, Inc. New York, Basle (eds. Woodrow, Levine), (1990) 85. Slow release from a subcutaneous depot site permits extensive. . . Slow release is favored by adsorbing antigens onto aluminum hydroxide ("alum precipitation"); placing antigens into water-in-oil emulsions; incorporating antigens into **liposomes**; and other similar manipulations. This method is conceptually close to the one described in section A.

SUMM If a particular **vaccine** is highly immunogenic, the adjuvant effect of this **vaccine**, and also the characteristics it may possess for guiding the response toward a particular immunological pathway, may "spill over" into. . . IgE responses. Pure proteins co-administered with the parasite extracts will also evoke an IgE response; see Nossal, G.J.V., New Generation **Vaccines**, Marcel Dekker, Inc. New York, Basle (eds. Woodrow, Levine), (1990) 85. Presumably, this effect is somehow connected to the production. . .

SUMM . . . and Smith, G. L., Macket, M. and Moss, B. Nature 302 (1983), 490, who genetically engineered the genome of the **vaccinia** virus to additionally include genes coding for important host-protective antigens of various pathogens. These are synthesized by the infected cell together with **vaccinia** virus particles and antigens. An improvement of this concept was introduced by Langford, C. J., Edwards, S. J., Smith, G.. . . gene encoding the soluble S antigen of Plasmodium falciparum, and inserted the resulting hybrid gene into the genome of a **vaccinia** virus. The construct caused a significantly enhanced immunogenicity. Live Salmonella, BCG and measles virus have also been successfully used for the expression of foreign antigen. Thus, the advantages of a live attenuated **vaccine** can be combined with those of a **vaccine** based on viruses containing recombinant DNA.

SUMM A further development of this idea is to insert genes for various interleukins into genetically engineered **vaccinia** viruses already carrying genes for important antigens. For example, the immune response to **vaccinia** virus itself can be markedly enhanced by the insertion of the IL-2 gene into the viral genome, permitting immunodeficient mice. .

SUMM . . . as saponin or quil A. Incubation in immunostimulating complexes (iscoms) have been used in a number of experimental and veterinary **vaccines**. They improved the immunogenicity of several antigens, especially of viral membrane proteins.

SUMM . . . its proinflammatory and encephalopathogenic potential. Surface-active agents display a number of side reactions: they are irritating, proinflammatory, they bind to **cholesterol** and lyse cells. Interleukins can provoke systemic reactions and therefore routine use in mass **vaccination** may be undesirable. Safety concerns prevented the use of genetically engineered microorganisms as carriers of genes for important antigens in. . . the antigen particulate often goes in parallel with a significant loss of the amount of antigen. The immunostimulatory effect of **liposome**-associated antigen on the humoral response is a widely recognized phenomenon, but immunopotentiation is limited and the mechanism by which this. . .

SUMM The solution to the above technical problem is achieved by providing the immunostimulating reconstituted influenza virosomes (IRIVs) and **vaccines** containing said IRIVs which are characterized in the claims. These IRIVs can be used as vehicles which actively transport desired. . .

SUMM . . . phospholipids or a mixture thereof. At least it contains two different compounds selected from the group of glycero-phospholipids, such as **phosphatidylcholine** or phosphatidylethanolamine, and **cholesterol**. **Phosphatidylcholine** and phosphatidylethanolamine are preferred, in particular in a ratio of 4:1. In preferred embodiments of the present invention, the ratio. . .

SUMM . . . influenza virus envelope's membrane part. In a preferred embodiment the essentially reconstituted functional virus envelopes exhibit the form of a **unilamellar** bilayer. An example of such a lacking component is the matrix protein of the natural influenza virus envelope.

SUMM . . . as a "recognition antigen" since most humans can be considered "primed" to HA due to prior exposure through disease or **vaccination**.

SUMM . . . all human beings have antibodies against influenza antigen. These antibodies originate either from a previous influenza infection or from a **vaccination**) speeds entry of antigens recognized by said antibodies not only into macrophages but also into lymphoid follicles, in which antigens. . .

SUMM . . . natural infection, by which said antigen is transported to lymphoid follicles and elsewhere in lymphatic tissue (Nossal, G.J.V., New Generation **Vaccines** (ed. Woodrow, G. C. and Levine, M. M.), Marcel Dekker, Inc., (1990) 85. The mechanism would be an adjunct to. . .

SUMM . . . bacteria or parasites, or a toxin. Examples of viruses are hepatitis A, B, C, D or E virus, Polio virus, **HIV**, Rabies virus, Influenza virus or Parainfluenza virus. Examples of bacteria are Pseudomonas, Klebsiella, E. coli, Salmonella typhi, Haemophilus influenzae, Bordetella. . .

SUMM In another embodiment, the present invention relates to a **vaccine** containing an IRIV of the present invention. Optionally, these **vaccines** additionally contain a suitable pharmaceutically acceptable carrier and/or diluent. These **vaccines** can be administered in conventional routes and dosages.

DRWD FIG. 3: Tolerance of hepatitis A **vaccines**. Comparison of IRIV-HAV **vaccines** versus Al-HAV **vaccines**.

DRWD FIG. 4: Immunogenicity of hepatitis A **vaccines**. Comparison of IRIV-HAV **vaccines** versus Al-HAV **vaccines**.

DETD (A) A dispersion of **phosphatidylcholine** (e.g. lecithin, SIGMA) (75%), phosphatidylethanolamine (SIGMA) (20%) and **cholesterol** (SIGMA) (5%) (all phospholipids 1-2% (w/v)=0.013-0.027M) in 0.1M NaCl containing 0.01M Tris/HCl, pH 7.3 was prepared by mixing these compounds. . .

DETD . . . an ultrasonification apparatus (Bransonic, Branson Europe BV, frequency 50 kHz±10%). 10 seconds of ultrasonic shocks repeated every minute yielded small **unilamellar** IRIVs. The sample volumes and column dimensions were such that a complete separation of IRIVs eluted at the void volume. . .

DETD **Phosphatidylcholine** (**PC**; Sigma Chemical Co., St. Louis, Mo.) and

phosphatidylethanolamine (PE, Sigma), (700:200 mg/mg), were suspended in 0.01M Tris--0.1M NaCl, pH 7.3,. . . fractions, which contained the IRIV, were pooled and re-chromatographed under identical conditions. The IRIV possessed an average diameter of approximately **150 nm**.

DETD Production of an HAV-IRIV **vaccine**

DETD . . . (Millipore). A preservative (thiomersal) was added to a final dilution of 10^{-4}. Aliquots of 0.6 ml of the final bulk **vaccine** were filled into **vaccine** vials under sterile conditions. Safety and potency tests were performed according to international regulations.

DETD Preparation of an anti-idiotype IRIV **vaccine** against hepatitis C

DETD . . . 125 C (1974), 373]. The major advantage of using anti-Id antibodies (Ab2) for eliciting antigen-specfic antibodies (Ab3) is that the **vaccine** recipient is never in contact with infectious agents or materials containing foreign genes.

DETD The anti-idiotype IRIV **vaccine** against hepatitis C was prepared as follows: Sheep were immunized with an Ab_1 (dissolved of a concentration of 1 mg/ml. . .

DETD . . . was diluted to a protein concentration of 50 μg/ml with PBS, pH 7.4, and portioned in 0.6 ml aliquots in **vaccine** vials.

DETD Safety and Immunogenicity of Inactivated Hepatitis A **Vaccines**: Comparision of IRIV-HAV prepared according to Example 1 with Alum-absorbed **Vaccine**

DETD . . . number ATCC CCL 171). The virus was inactivated by treatment with formaldehyde (0.05%) at 37° C. for 10 days. Two **vaccine** series were tested. **Vaccine** series 1 consisted of inactivated virus linked to IRIVs according to Example 1 (A) (IRIV-HAV). **Vaccine** of series 2 was an alumadsorbed preparation containing 0.4% $Al(OH)_3$ (Al-HAV). Both **vaccines** contained 150 ng of HAV antigen per 0.5 ml dose. Seronegative adult volunteers (two groups of 15 persons each) received. . . chemistry were detected. With respect to local reactions, IRIV preparations provoked a significantly lower percentage of reactions than the alum-absorbed **vaccine**. The results of these experiments are summarized in FIG. 3.

DETD . . . the range of the anti-HAV antibody titer. Thus, the range of the anti-HAV antibody titers for the IRIV and alum-adsorbed **vaccine** formulations on day 21 was 82-988 and 69-844, respectively. The geometric mean titer (range) for the IRIV and alum-adsorbed **vaccine** formulations on day 28 was 453 mIU/ml (92-1210) and 361 mIU/ml (60-929), respectively. Thus, the IRIV preparations of the present invention are superior to alum-adsorbed **vaccines**.

DETD . . . a phase I clinical study with 120 human volunteers it could be demonstrated that one single IRIV adjuvanted hepatitis A **vaccine** dose induced protective antibody titers against hepatitis A which were 7 times higher than the antibody titer after the alum. . .

DETD A total of 120 HAV seronegative (<10 mIU/ml) healthy adults were randomized to receive either fluid, alumadsorbed, or IRIV **vaccine** according to Example 1 (B). The **vaccine** (0.5 ml) was administered intramuscularly into the deltoid region. Volunteers were observed for approximately 30 minutes after **vaccination** for immediate-type reactions. Each volunteer was asked to record all adverse reactions on a report sheet for the 4 days. . . following immunization. Serum samples for anti-HAV antibody determinations were taken at the time of immunization and 14 days later. Each **vaccine** formulation contained 1 μg of HAV antigen per 0.5 ml dose. One dose of the IRIV-HAV formulation also contained 10 μg of influenza HA and 125 μg total phospholipids. All three **vaccines** were found to be sterile and nontoxic for animals by standard test methods. In addition, all 3 formulations elicited a. . .

DETD . . . immunization are shown in Table I. Pain at the injection site was the most frequently reported complaint with all the **vaccines**. Such discomfort was classified as moderate by one **vaccine** (2.5%) who received the fluid formulation, 9 (23%) who were immunized with the alum-adsorbed **vaccine**, and one (2.5%) who received the IRIV preparation. Severe pain was reported by one subject who received the alum-adsorbed **vaccine**. All other subjects who reported a "painful"

vaccine was associated with a significantly (P<0.01) higher incidence of both pain and swelling/induration as compared to either the fluid or IRIV formulations. No systemic reactions attributable to **vaccination** were noted.

DETD The anti-HAV antibody response engendered 14 days after **vaccination** is shown in Table II. Immunization with the fluid **vaccine** yielded a geometric mean titer (GMT) of 15.7 mIU/ml with 30% of subjects seroconverting (≥20 mIU/ml). While the alum-adsorbed **vaccine** induced both a modestly higher GMT (21.3 mIU/ml) and seroconversion rate (44%), neither was significantly greater than that obtained with the fluid **vaccine**. In contrast, the IRIV **vaccine** formulation elicited a far more vigorous antibody response. The GMT of 139.8 was significantly (P<0.0001) higher compared to either of the other two **vaccines**. All but one **vaccine** possessed ≥100 mIU/ml. Of greater importance was the fact that all **vaccines** seroconverted by day 14 compared to less than 50% for the other **vaccine** formulations (P<0.005).

DETD TABLE I

Adverse Reactions Associated with Immunization

	Local reactions (%)		Systemic reactions (%)			
Vaccine	Pain	Swelling/ Induration	Red-ness	Fever	Head-ache	Malaise
Fluid	42*	0.parallel.	0	0	0	0
$Al(OH)_3$ -adsorbed	88+	23.paragraph.	0	0	0	0
IRIV	25.sctn.					

. . .

DETD TABLE II

Immunogenicity of Fluid, $Al(OH)_3$ -Adsorbed, and IRIV-Adjuvanted Hepatitis A **Vaccines**

Vaccine Formulation	Geometric mean titer (range) in mIU/ml Day 0	Day 14	Seroconversion rate No. ≥20 mIU/total (%)
Fluid	<10	15.7 (<10-100)*	12/40 (30%).parallel.
$Al(OH)_3$ -adsorbed	<10	21.3 (10-100)+	18/40 (44%).paragraph.
IRIV-adjuvanted	<10	139.8 (25-300).sctn.	40/40 (100%)**

Subjects received a single dose of **vaccine** on day 0.

.sctn. vs * or + : P < 0.0001

**vs .parallel. or .paragraph. : P < 0.005

. . . A virus (HAV), said virus being attached or adsorbed to the surface of the IRIV; wherein the IRIV is substantially **unilamellar**.

. . . 3. The IRIV according to claim 1, wherein said essentially reconstituted functional virus envelopes are in the form of a **unilamellar** bilayer.

5. The IRIV according to claim 1, wherein said mixture comprises **phosphatidylcholine** and phosphatidylethanolamine.

6. The IRIV according to claim 5, wherein **phosphatidylcholine** and phosphatidylethanolamine are in a weight ratio of 4:1.

8. A **vaccine** containing an IRIV according to claim 1 optionally in combination with a suitable pharmacologically acceptable carrier and/or diluent.

L27 ANSWER 7 OF 8 USPATFULL on STN
93:84900 Artificial viral envelopes.
Schreier, Hans, Gainesville, FL, United States
Chander, Ramesh, Bombay, India
Stecenko, Arlene A., Gainesville, FL, United States
Univ. of Florida Research Foundation, Inc., Gainesville, FL, United States (U.S. corporation)
US 5252348 19931012
APPLICATION: US 1992-923016 19920730 (7) <--
DOCUMENT TYPE: Utility; Granted.

AB The production of artificial viral envelopes by a novel double-detergent dialysis technique is disclosed. Specifically exemplified is the production of **HIV**-1 and RSV viral envelopes. The resulting artificial viral envelopes are essentially identical to the natural virus with regard to characteristics which are relevant to immunogenicity.

CLM What is claimed is:
1. A two-step process for preparing lipid vesicles having a size of about **150 nm** to about 300 nm and an approximately 1:1 **cholesterol**:phospholipid ratio characteristic of natural viruses and having antigenic or immunogenic proteins or peptides on the outer surface of said vesicle; said first step comprising solubilizing phospholipids and **cholesterol** in a sodium cholate detergent solution such that the molar ratio of detergent to total lipids is about 45:1, and the ratio of phospholipid to **cholesterol** is about 1:1, said first step further comprising the removal of said detergent by dialysis to obtain a rigid vesicle, said second step comprising insertion of proteins or peptides into the outer membrane of said vesicle, wherein the insertion of said proteins or peptides comprises mixing for about one hour said rigid vesicle from said first step with an aqueous solution of deoxycholate detergent, wherein the ratio of detergent to lipids is about 8:1, said process further comprising the addition of said proteins to said mixture of the deoxycholate detergent and the vesicle, followed by the removal of said detergent by dialysis.

2. The process, according to claim 1, wherein viral core proteins are incorporated into said lipid vesicle.

3. The process, according to claim 1, wherein said proteins or peptides are glycoproteins.

4. The process, according to claim 1, which further comprises a final step wherein said vesicles are freeze-dried.

5. The process, according to claim 3, wherein said glycoproteins are either natural or recombinant proteins from the human immune deficiency virus or the respiratory syncytial virus.

AI US 1992-923016 19920730 (7) <--

AB The production of artificial viral envelopes by a novel double-detergent dialysis technique is disclosed. Specifically exemplified is the production of **HIV**-1 and RSV viral envelopes. The resulting artificial viral envelopes are essentially identical to the natural virus with regard to characteristics. . .

SUMM Enormous efforts are currently being made to develop a **vaccine** against **HIV**-1 (Laurence, J. [1990] AIDS Res. 6:175-181). Currently tested systems employ either killed virus, stripped of its coat, or one of the **HIV** proteins, either surface glycoproteins (gp120, gp160) or core proteins (p24, hgp30). The main limitation with subunit protein

vaccines is their poor immunogenicity, even if combined with an adjuvant, e.g., muramyltripeptide or others.

SUMM Another approach, used by several investigators, has been to enhance the immunogenic activity of subunit **vaccines** by preparing protein-containing lipid vesicles, called "proteoliposomes," "immunoliposomes," "immunosomes," "virosomes," or the like. Methods to prepare these vesicles vary widely,. . .

SUMM . . . J. Virology 63:2951-2958; El Guink, N., R. M. Kris, G. Goodman-Snitkoff, P. A. Small Jr., and R. J. Mannino [1989] **Vaccine** 7:147-151; Gould-Fogerite, S., J. E. Mazurkiewicz, D. Bhisitkul, and R. J. Mannino [1988] In Advances in Membrane Biochemistry and Bioenergetics. . .

SUMM Thibodeau et al. (1989, supra) describe a method of "anchoring" **HIV** gp160 on the surface of **liposomes** to prepare "**HIV**-immunosomes." However, the **liposome** composition is not disclosed, and the "anchoring" is achieved by simple incubation of preformed **liposomes** with the purified gp160.

SUMM In contrast to Thibodeau et al.'s (1989, supra) approach, we have generated a viral envelope identical to the **HIV**-1 envelope with respect to its lipid composition, an approximately equimolar lipid: **cholesterol** ratio, unilamellarity, and vesicular size. Furthermore, the incorporation of gp160 was achieved by partial re-solubilization of the lipid envelopes in. . . hydrophobic gp41 part of gp160 into the lipid envelope. Using our innovative approach, it is now possible to formulate subunit **vaccines** which are superior to conventional **vaccines**.

SUMM Disclosed are novel artificial viral envelopes, essentially identical to natural viral envelopes, e.g., to the **human immunodeficiency virus** (**HIV**-1), the respiratory syncytial virus (RSV), or other viruses, and a novel method, double-detergent dialysis, essential to prepare same.

SUMM Natural viral envelopes are unique in their **cholesterol**:phospholipid ratio of about 0.8-1.2 and, until now, could not be reproduced by any known preparation technique.

SUMM The artificial viral envelopes can be characterized by: (i) an advantageous phospholipid:**cholesterol** ratio of about 1:1; (ii) a virus-specific phospholipid composition; (iii) a homogenous size distribution around 250 nm, similar to natural viral size; (iv) a uniquely stable, rigid, **unilamellar** structure; (v) envelope glycoproteins such as the **HIV**-1 gp160, RSV G (aggregation) and F (fusion) proteins, and others, inserted in the outer surface; (vi) high fusogenic activity; (vii). . .

SUMM The following applications of the artificial viral envelopes are proposed: (i) synthetic subunit **vaccines**; (ii) highly targetable and fusogenic drug delivery devices for delivery of antiviral agents to infected cells; (iii) highly specific cell. . .

SUMM The artificial lipid vesicles of the subject invention are characterized by: (i) a **cholesterol**:phospholipid ratio of about 0.8 to about 1.2, similar to that of natural viral envelopes; (ii) a phospholipid composition similar to. . . 50 to about 500 nm, which is similar to that of the natural viral particle; and (iv) a physically stable **unilamellar** membrane structure. In one preferred embodiment of the invention, the novel lipid vesicles may be further characterized by (v) envelope proteins such as the **HIV**-1 gp160, RSV G (aggregation) and F (fusion) proteins, and others, inserted in the outer surface; (vi) high fusogenic activity; (vii). . .

SUMM Preferably, the phospholipid composition of the synthetic viral envelopes should be similar to the natural viral composition and should comprise **phosphatidylcholine** (**PC**), phosphatidylserine (PS), phosphatidylethanolamine (PE), and sphingomyelin (SM). The envelope may further comprise additional lipids such as phosphatidylinositol.

SUMM . . . a novel method--double detergent dialysis. As specifically exemplified herein, this method consists essentially of two steps: (1) preparation of the phospholipid/**cholesterol** envelope by solubilization of the lipids and **cholesterol** with sodium cholate or other appropriate detergent as the solubilizing agent at a unique molar ratio of approximately 45:1, followed. . .

SUMM . . . may be, for example, from about 1×10^6 :1 or higher

unilamellar, however, oligolamellar may also be acceptable for some purposes.

SUMM An important aspect of the double-detergent dialysis method is that the two steps are independent processes. During the first step, **unilamellar** lipid envelopes in a size range of about 50 to about 500 nm or, preferably, about 150 to about 350. . .

SUMM . . . insertion of the protein onto the envelope. The use of freeze-drying procedures can reduce or eliminate the need for keeping **vaccines** refrigerated and, therefore, can be very important for field uses, especially in underdeveloped countries. The stability of the lipid vesicles. . .

SUMM . . . any one of a number of viruses, bacteria, and parasites. Specifically exemplified herein is the construction of artificial RSV and **HIV**-1 envelopes. The following applications of an artificial **HIV**-1 viral envelope can be envisioned:

SUMM 1. Artificial **HIV**-1 envelopes, being identical to the natural **HIV**-1 envelopes in size and chemical composition, would induce both a humoral and cellular immune response; thus, they may serve as a synthetic subunit **vaccine** against **HIV**-1. Several significant advantages of such a synthetic **vaccine** over **vaccination** with a natural modified **HIV** preparation can immediately be appreciated: (a) since the artificial **HIV**-1 envelope is void of genetic information, there is no danger of infection; (b) the synthetic **vaccine** is likely to be more immunogenic due to proper presentation of the antigen to lymphocytes; (c) insertion of desired composite epitopes will yield highly efficacious **vaccines**; and (d) antigenic drift, once identified and reproduced by recombinant techniques, can immediately be simulated with the synthetic **vaccine**.

SUMM 2. Due to the unique capability of the **HIV**-1 virus to bind to and fuse with CD4+ cells, the artificial **HIV**-1 envelope may be utilized as a novel "target-seeking" drug delivery system for antiviral drugs for delivery of antiviral agents specifically to **HIV**-1 infected cells. This is an important and novel aspect of the artificial **HIV**-1 envelope since both the antiviral killing efficacy of antiviral drugs and stimulation of production of virus-neutralizing antibodies can be combined. . .

SUMM 3. The investigation of biochemical and immunological pathways as well as the development of **vaccines** and antiviral drugs is complicated by restrictive regulations necessary to protect personnel from inadvertent exposure to **HIV**-1. Since the artificial **HIV**-1 envelope does not contain genetic information, its use would be much less restricted. Therefore, the artificial **HIV**-1 envelope may serve (a) as an in vitro model for viral infectivity, particularly to investigate viral cell binding and cell. . .

SUMM Artificial RSV envelopes may serve as a synthetic **vaccine** against RSV in a similar fashion as described above for **HIV**-1. In addition, it may serve as a drug delivery system for antiviral agents such as ribavirin and others for the. . .

SUMM . . . virus; measles virus; mumps virus; rabies and rabies-related viruses; retroviruses including human T-lymphotrophic virus type I and II (HTLV-I/II) and **human immunodeficiency virus** type 1 and 2 (**HIV**-1/2); rhinoviruses; rubella virus; orthopoxvirus group including smallpox virus; B19 parvovirus; human papilloma viruses; Newcastle disease virus; Semliki Forest virus; encephalomyocarditis. . .

SUMM In addition to their uses as subunit **vaccines** and vectors, the envelopes described herein can be used in a variety of therapeutic applications including the destruction of viruses. . .

SUMM Phospholipids were purchased from the following sources; egg **phosphatidylcholine** (**PC**) (lot #37F-8420), phosphatidylserine (PS) (lot #99F-83561) from bovine brain, egg phosphatidylethanolamine (PE) (lot #58F-8371), **cholesterol** from porcine liver (lot #36F-7040), deoxycholic acid (lot #108F-0331) and sodium cholate (lot #78F-0533) were from Sigma Chemical Co., St.. . .

SUMM **HIV**-1 gp160 envelope protein (lot #8962R-1) at a concentration of 100 μg/ml in 5 mM Tris buffer containing 0.005% polysorbate 20. . .

Haven, Conn.). According to the manufacturer's specifications, it is a full-length glycosylated recombinant protein derived from the env gene of **HIV**-1. The protein is produced in insect cells using the baculovirus expression system and purified by low pressure, low temperature chromatography.

DETD Preparation of Artificial **HIV**-1 or RSV Envelopes (Without Protein)

DETD Stock lipid solutions were prepared as shown in Table 1. Briefly, enough **cholesterol** or phospholipids were dissolved individually in 10 ml chloroform to give the concentrations indicated in Table 1. Sodium cholate stock. . .

DETD TABLE 1

Lipid Composition and Stock Solutions

	mg/10 ml	μmoles/	mole % of
	$HCCl_3$		
	MW	10 ml	total PL
cholesterol (CH)	38	386	98.4
phosphatidylcholine (**PC**)	20	786	25.4
			23.7
phosphatidylethanolamine (PE)	18	743	24.2
			22.6
phosphatidylserine (PS)	23	832	27.6
			25.7
sphingomyelin (SM)	22	731	30.1
			28.1

TOTAL PHOSPHOLIPID. . .

DETD As shown in Table 2, the ratio of total phospholipid:**cholesterol** was approximately 1, and the detergent:total lipid ratio was approximately 45.

DETD TABLE 2

Lipid:**Cholesterol** and Lipid:Detergent Ratio

CONC. (μMOLE/10 ml)		MOLAR RATIO
Total Phospholipid	**Cholesterol**	
initial		
107.3	98.4	0.92
recovered after dialysis		
79.4 (74.0%)	69.2 (70.3%)	0.87
Total Lipid	Sodium Cholate[1]	
205.7	9289.4	45.2

[1] Total amount of detergent used:. . .

DETD The phospholipid composition of artificial and natural **HIV**-1 envelopes (Gordon, L. M., F. C. Jensen, C. C. Curtain, P. W. Mobley, R. C. Aloia (1988) In Lipid Domains. . .

DETD TABLE 3

Lipid Composition of Artificial and Natural **HIV**-1 Envelope

	MOLE % OF TOTAL PHOSPHOLIPIDS						
LIPID	**PC**	PE	SM	PS	PI[2]	PA[3]	Other
NATURAL[1]	23.8	24.6	28.3	15.1	2.1	0.9	5.0
ARTIFICIAL							

n.a. n.a.

. . .

DETD The ultrastructure of the vesicles was determined by freeze-fracture electron microscopy. The results of the electron microscopy showed perfectly **unilamellar** artificial envelopes.

DETD **Cholesterol** was determined according to the method of Zlatkis et al. (Zlatkis, A., B. Zak, and A. J. Boyle [1953] J.. . .

DETD The final phospholipid:**cholesterol** ratio was 0.87, only slightly different than the original ratio of 0.92.

DETD Stability of Artificial **HIV** Envelopes (Without Protein)

DETD Artificial **HIV**-1 envelopes (without protein) were stored at 4° C. in the refrigerator. Average size and size distribution were analyzed periodically by. . . shown in Table 4. Such practically ideal physical stability was a completely unexpected finding, since it is known that conventional **liposomes** of a comparable size and high **cholesterol** content "grow" upon storage due to the high free energy content of the highly curved membranes.

DETD TABLE 4

Physical Stability of Artificial **HIV**-1 Envelopes

Time from Prep. (days)	Average Size (nm) ± Size Distribution Sample 1	Sample 2	Sample 3	Sample 4
0	267 ± 137			

. . .

DETD Preparation of Artificial **HIV**-1 Envelopes with gp160

DETD Complete artificial **HIV**-1 envelopes containing the **HIV**-1 gp160 were prepared by a novel double-detergent dialysis technique. Double-detergent dialysis is a requirement because the high concentration of **cholesterol** in the lipid envelope requires a high concentration of detergent. Therefore, the dialysis is performed in two steps. The first. . .

1. A two-step process for preparing lipid vesicles having a size of about **150 nm** to about 300 nm and an approximately 1:1 **cholesterol**:phospholipid ratio characteristic of natural viruses and having antigenic or immunogenic proteins or peptides on the outer surface of said vesicle; said first step comprising solubilizing phospholipids and **cholesterol** in a sodium cholate detergent solution such that the molar ratio of detergent to total lipids is about 45:1, and the ratio of phospholipid to **cholesterol** is about 1:1, said first step further comprising the removal of said detergent by dialysis to obtain a rigid vesicle,. . .

L27 ANSWER 8 OF 8 USPATFULL on STN

93:54529 Microreservoir **liposome** composition and method.

Martin, Francis J., San Francisco, CA, United States
Woodle, Martin C., Menlo Park, CA, United States
Redemann, Carl, Walnut Creek, CA, United States
Yau-Young, Annie, Palo Alto, CA, United States
Radhakrishnan, Ramachandran, Fremont, CA, United States
Liposome Technology, Inc., Menlo Park, CA, United States (U.S. corporation)

US 5225212 19930706

APPLICATION: US 1990-624548 19901210 (7) <--

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A **liposome** composition for extended release of a therapeutic compound into the bloodstream. The **liposomes** are composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with hydrophilic polymer, have sizes in a selected size range between 0.1 and 0.4 microns, and contain the therapeutic compound in **liposome**-entrapped form. The dosage form of the composition contains at least about three times the dose of the compound required

extending to at least 24 hours the period in which an intravenously administered therapeutic compound is therapeutically active in the bloodstream, and novel **liposomes** compositions for practicing the method.

CLM What is claimed is:

1. A **liposome** composition effective to extend, to at least 24 hours, the period of effective activity of a therapeutic compound which can be administered intravenously in a therapeutically effective amount and which is cleared in free form from the bloodstream with a halflife of less than about 4 hours, comprising **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with a polymer selected from the group consisting of polyethyleneglycol, polyacetic acid and polyglycolic acid, and (ii) having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound in **liposome**-entrapped form, for intravenous administration at a dose of the composition which contains an amount of the compound in **liposome**-entrapped form which is at least three times such therapeutically effective amount.

2. The composition of claim 1, wherein the hydrophilic polymer is polyethyleneglycol having a molecular weight between about 1,000-5,000 daltons.

3. The composition of claim 2, wherein the polymer is derivatized to a phospholipid.

4. The composition of claim 1, wherein the polymer is selected from the group consisting of polyacetic acid and polyglycolic acid.

5. A **liposome** composition effective to extend, to at least 48 hours, the period of therapeutic activity of a polypeptide which can be administered intravenously in a therapeutically effective amount, which is cleared in free form from the bloodstream with a halflife of less than about 4 hours, and whose therapeutically active blood concentration is in the picogram-nanogram/ml concentration range, comprising **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with a polymer selected from the group consisting of polyethyleneglycol, polyacetic acid and polyglycolic acid, and (ii) having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the polypeptide in **liposome**-entrapped form, for intravenous administration at a dose of the composition which contains an amount of the polypeptide **liposome**-entrapped form which is at least three times such therapeutically effective amount.

6. The composition of claim 5, wherein the hydrophilic polymer is polyethyleneglycol having a molecular weight between about 1,000-5,000 daltons.

7. The composition of claim 5, wherein the polypeptide is a peptide hormone which is therapeutically active at a plasma concentration in the picogram/ml range, and the **liposome** composition is effective to release the hormone in a therapeutically effective dose for a period of at least five days after intravenous administration of the composition.

8. The composition of claim 7, wherein the peptide hormone is vasopressin.

9. The composition of claim 5, wherein the compound is a protein selected from the group consisting of superoxide dismutase, glucocerebrosidase, asparaginase, adenosine deaminase, interferons (alpha, beta, and gamma), interleukin (1,2,3,4,5,6,7), tissue necroses factor (TNF-alpha, beta), colony stimulating factors (-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**,

[illegible] vaccine, and melanoma vaccine, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha, beta), somatomedin C (IGF-1), and a ribosome inhibitor protein.

10. The composition of claim 9, wherein the protein is macrophage colony stimulating factor.

11. A method of extending, to at least 24 hours, the period of effective activity of a therapeutic compound which can be administered intravenously in a therapeutically effective amount, and which has a halflife in the bloodstream in free form of less than about 4 hours, comprising providing a **liposome** composition containing **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with ah polymer selected from the group consisting of polyethyleneglycol, polyacetic acid and polyglycolic acid, and (ii) having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound at least about 70% in **liposome**-entrapped form, and administering the **liposome** composition intravenously to a subject at a dose which contains an amount of the compound which is at least three times such therapeutically effective amount.

12. The method of claim 11, wherein the hydrophilic polymer is polyethyleneglycol having a molecular weight between about 1,000-5,000 daltons.

13. The method of claim 11, wherein the polymer is selected from the group consisting of polylactic acid and polyglycolic acid.

14. The method of claim 11, wherein the compound is a peptide hormone which is therapeutically active at a plasma concentration in the picogram-to-nanogram/ml range, and said administering is effective to release the hormone in a therapeutically effective dose for a period of at least five days.

15. The method of claim 14, wherein the peptide hormone is vasopressin.

16. The method of claim 11, wherein the compound is a protein selected from the group consisting of superoxide dismutase, glucocerebrosidase, asparaginase, adenosine deaminase, interferons (alpha, beta, and gamma), interleukin (1,2,3,4,5,6,7), tissue necroses factor (TNF - alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha, beta), somatomedin C (IGF-1), and a ribosome inhibitor protein.

17. The method of claim 16, wherein the protein is macrophage colony stimulating factor.

18. A **liposome** composition effective to extend, to at least one week, the period of effective activity of a therapeutic compound which can be administered in a therapeutically effective amount, comprising **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with a polymer selected from the group consisting of polyethyleneglycol, polyacetic acid and polyglycolic acid, and (ii) having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound in **liposome**-entrapped form, for subcutaneous administration at a dose of the composition which contains an amount of the compound in **liposome**-entrapped form which is at least ten times such therapeutically effective intravenously administered amount.

19. The composition of claim 18, wherein the compound is a polypeptide

selected from the group consisting of superoxide dismutase, glucocerebrosidase, asparaginase, adenosine deaminase, interferons (alpha, beta, and gamma), interleukin (1,2,3,4,5,6,7), tissue necrosis factor (TNF - alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha, beta), somatomedin C (IGF-1), and a ribosome inhibitor protein.

20. The composition of claim 19, wherein the polypeptide is vasopressin.

21. A method of extending, to at least one week, the period of effective activity of a therapeutic compound which can be administered in a therapeutically effective amount, comprising providing a **liposome** composition containing **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with a polymer selected from the group consisting of polyethyleneglycol, polyacetic acid and polyglycolic acid, and (ii) having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound at least about 70% in **liposome**-entrapped form, and administering the composition subcutaneously to a subject at a dose which contains an amount of the compound in **liposome**-entrapped form which is at least ten times such therapeutically effective intravenously administered amount.

22. The method of claim 21, wherein the compound is a peptide hormone selected from the group consisting of superoxide dismutase, glucocerebrosidase, asparaginase, adenosine deaminase, interferons (alpha, beta, and gamma), interleukin (1,2,3,4,5,6,7), tissue necroses factor (TNF-alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha, beta), somatomedin C (IGF-1), and a ribosome inhibitor protein.

23. The method of claim 22, wherein the polypeptide is vasopressin.

24. A **liposome** composition composed of vesicle-forming lipids and a vesicle-forming lipid derivatized with a hydrophilic polymer selected from the group consisting of polylactic acid and polyglycolic acid.

25. A lipid composition composed of a vesicle-forming lipid having a polar head group, and a polylactic acid moiety derivatized to said head group.

26. A lipid composition composed of a vesicle-forming lipid having a polar head group, and a polyglycolic acid moiety derivatized to said head group.

TI Microreservoir **liposome** composition and method

AI US 1990-624548 19901210 (7) <--

AB A **liposome** composition for extended release of a therapeutic compound into the bloodstream. The **liposomes** are composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with hydrophilic polymer, have sizes in a selected size range between 0.1 and 0.4 microns, and contain the therapeutic compound in **liposome**-entrapped form. The dosage form of the composition contains at least about three times the dose of the compound required for. . . least 24 hours the period in which an intravenously administered therapeutic compound is therapeutically active in the bloodstream, and novel **liposomes** compositions for practicing the method.

SUMM The present invention relates to a **liposome** composition and method for

administering a therapeutic compound into the bloodstream over an extended period.

SUMM Gabizon, A. Huberty, J. Straubinger, R. and Papahadjopoulos, D. (1988-1989) J. **Liposome** Resh. 1, 123-135.

SUMM Poste, G., et al., in "**Liposome** Technology" Volume 3, page 1 (Gregoriadis, G., et al, eds.), CRC Press, Boca Raton (1984);

SUMM . . . produced through recombinant DNA technology, such as human growth hormone, human insulin, α-interferon, interleukin-2, TPA, and a variety of peptide **vaccines**, all of which are now commercially available (Banga). As oral administration generally does not result in therapeutic responses, the parenteral. . .

SUMM **Liposomes** have been proposed as a carrier for intravenously (IV) administered compounds. However, the use of **liposomes** for slow release of **liposome**-entrapped material into the bloodstream has been severely restricted by the rapid clearance of **liposomes** from the bloodstream by cells of the reticuloendothelial system (RES). Typically, the RES will remove 80-95% of IV injected **liposomes** within one hour, and effectively remove circulating **liposomes** from the bloodstream within of 4-6 hours.

SUMM A variety of factors which influence the rate of RES uptake of **liposomes** have been reported (e.g., Gregoriadis, 1974; Jonah; Gregoriadis, 1972; Juliano; Allen, 1983; Kimelberg, 1976; Richardson; Lopez-Berestein; Allen, 1981; Scherphof; Gregoriadis,. . . 1983; Senior, 1985; Allen, 1983; Ellens; Senior, 1982; Hwang; Ashwell; Hakomori; Karlsson; Schauer; Durocher; Greenberg; Woodruff; Czop; and Okada). Briefly, **liposome** size, charge, degree of lipid saturation, and surface moieties have all been implicated in **liposome** clearance by the RES. However, no single factor identified to date has been effective to provide long blood halflife, and more particularly, a relatively high percentage of **liposomes** in the bloodstream than 1 day or more after IV administration.

SUMM One factor which does favor longer **liposome** lifetime in the bloodstream is small **liposome** size, typically in the size range of small **unilamellar** vesicles (SUVs): 0.03-0.07 microns. However, the intravesicular volume of SUVs is quite limited, to the extent that loading SUVs with. . .

SUMM It is therefore one general object of the invention to provide a **liposome** composition and method for administering a therapeutic compound for an extended period in the bloodstream.

SUMM The invention includes, in one aspect, a **liposome** composition effective to extend to at least 24 hours, the period of effective activity of an therapeutic compound which can. . . effective amount, and which has a blood halflife, in free form, of less than about 4 hours. The composition includes **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with a biocompatible hydrophilic polymer,. . . having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound in **liposome**-entrapped form. The composition is intended for intravenous administration at a dose which contains an amount of the **liposome**-entrapped compound which is at least three times the therapeutically effective dose for the compound in free form.

SUMM . . . necrosis factor (TNF - alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, insulin-like growth factor, nerve growth factor, platelet-derived growth factor, tumor. . .

SUMM . . . which has a halflife in the blood, in free form, of less than about 4 hours. In this method, a **liposome** composition of the type described above is administered intravenously to a subject at a dose which contains an amount of. . .

SUMM Also disclosed is a **liposome** composition effective to extend to at least one week, the period of effective activity of an therapeutic compound which can be administered intravenously in a therapeutically

vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with a biocompatible hydrophilic polymer,. . . and (ii) having a selected mean particle diameter in the size range between about 0.07-0.15 microns, and the compound in **liposome**-entrapped form. The composition is intended for subcutaneous administration at a dose which contains an amount of the **liposome**-entrapped compound which is at least ten times such therapeutically effective intravenously administered amount.

SUMM The **liposome** composition is used in a method for extending the period of release of a therapeutic compound, preferably a polypeptide, in. . .

SUMM In another aspect, the invention includes a **liposome** composition composed of vesicle-forming lipids and a vesicle-forming lipid derivatized with polylactic acid or polyglycolic acid, and a lipid composition. . .

DRWD FIG. 7 is a plot of **liposome** retention time in the blood, expressed in terms of percent injected dose as a function of hours after IV injection, for PEG-PE **liposomes** containing different amounts of phosphatidylglycerol;

DRWD FIG. 8 is a plot similar to that of FIG. 7, showing retention times in the blood of **liposomes** composed of predominantly unsaturated phospholipid components;

DRWD FIG. 9 is a plot similar to that of FIG. 7, showing retention times in the blood of PEG **liposomes** (solid triangles) and conventional **liposomes** (solid circles);

DRWD FIG. 10 is a plot of blood lifetimes of PEG-**liposomes** sized by extrusion through 0.1 micron (solid squares), 0.2 micron (solid circles), and 0.4 micron (solid triangles) polycarbonate membranes;

DRWD FIG. 11 is a plot of blood retention times in **liposomes** containing a vesicle-forming lipid derivatized with polylactic acid (solid squares) and polyglycolic acid (open triangles);

DRWD . . . in rats, as a percentage of predosage rate, after surgery and IV administration of saline (control, open circles) and of PEG-**liposomes** containing entrapped vasopressin at total doses of 2 μg (closed squares), 8 μg (closed tiangles), and 24 μg (closed circles);

DRWD . . . in rats, as a percentage of predosage rate, after surgery and IV administration of saline (control, open circles) and of PEG-**liposomes** containing entrapped vasopressin at a total dose of 8 μg and mole percent of **cholesterol** in the **liposomes** of 33% (closed circles), 16% (closed triangles), and 0% (closed squares);

DRWD FIG. 15 shows the blood clearance kinetics of free macrophage-colony stimulating factor (M-CSF) (solid triangles), PEG-**liposomes** containing 30 mole percent **cholesterol** (solid triangles), and M-CSF associated with the PEG-**liposomes** (solid circles);

DRWD FIG. 16 shows the blood clearance kinetics of free M-CSF (solid triangles), **cholesterol**-free PEG-**liposomes** (solid triangles), and M-CSF associated with the PEG-**liposomes** (solid circles);

DRWD FIG. 17 is a plot of percent release of M-CSF into the blood from PEG **liposomes** containing 30 (solid circles) and 0 (solid triangles) mole percent **cholesterol**;

DRWD . . . as a percentage of predosage rate, after surgery and subcutaneous administration of saline (control, open circles) and vasopressin entrapped in PEG-**liposomes**, in an amount 25 μg (solid triangles), 100 μg (solid, circles), and 400 μg (solid diamonds); and

DRWD FIGS. 19A and 19B show the change in PEG-**liposome** size, as a function of homogenization time, for **liposome** particles in an homogenized suspension.

DETD . . . two hydrocarbon chains, typically acyl chains, and a polar head group. Included in this class are the phospholipids, such as **phosphatidylcholine** (**PC**), PE, phosphatidic acid (PA), phosphatidylinositol (PI), and sphingolipids such as sphingomyelin (SM), where the two hydrocarbon chains are typically between. . .

DETD Another vesicle-forming lipid which may be employed is **cholesterol** and

to a lipid bilayer membrane, particularly when derivatized with a high molecular weight polyalkylether, and therefore be less effective in promoting **liposome** evasion of the RES in the bloodstream.

DETD . . . moiety oreinted toward the exterior, polar surface of the membrane. An example of a latter type of vesicle-forming lipid is **cholesterol** and **cholesterol** derivatives, such as **cholesterol** sulfate and **cholesterol** hemisuccinate.

DETD . . . rigidity in a lipid bilayer structure and also contribute to greater bilayer stability in serum. Other lipid components, such as **cholesterol**, are also known to contribute to membrane rigidity and stability in lipid bilayer structures. Phospholipids whose acyl chains have a. . .

DETD . . . be used to join an activated polyalkylether to the hydroxyl group of an amphipathic lipid, such as the 5'-OH of **cholesterol**. Other reactive lipid groups, such as an acid or ester lipid group may also be used for coupling, according to. . .

DETD . . . with either polylactic acid or polyglycolic acid form part of the invention herein. Also forming part of the invention are **liposomes** containing these derivatized lipids, in a 1-20 mole percent.

DETD II. Preparation of **Liposome** Composition

DETD The lipid components used in forming the **liposomes** of the invention may be selected from a variety of vesicle-forming lipids, typically including phospholipids and sterols. As will be seen, one requirement of the **liposomes** of the present invention is long blood circulation lifetime. It is therefore useful to establish a standardized measure of blood. . .

DETD One method used for evaluating **liposome** circulation time in vivo measures the distribution of IV injected **liposomes** in the bloodstream and the primary organs of the RES at selected times after injection. In the standardized model which is used herein, RES uptake is measured by the ratio of total **liposomes** in the bloodstream to total **liposomes** in the liver and spleen, the principal organs of the RES. In practice, age and sex matched mice are injected intravenously (IV) through the tail vein with a radiolabeled **liposome** composition, and each time point is determined by measuring total blood and combined liver and spleen radiolabel counts, as detailed. . .

DETD Since the liver and spleen account for nearly 100% of the initial uptake of **liposomes** by the RES, the blood/RES ratio just described provides a good approximation of the extent of uptake from the blood to the RES in vivo. For example, a ratio of about 1 or greater indicates a predominance of injected **liposomes** remaining in the bloodstream, and a ratio below about 1, a predominance of **liposomes** in the RES. For most of the lipid compositions of interest, blood/RES ratios were calculated at 1, 2, 3, 4,. . .

DETD The **liposomes** of the present invention include 1-20 mole percent of the vesicle-forming lipid derivatized with a hydrophilic polymer, described in Section I. According to one aspect of the invention, it has been discovered that blood circulation halflives in these **liposomes** are largely independent of the degree of saturation of the phospholipid components making up the **liposomes**. That is, the phospholipid components may be composed of predominantly of fluidic, relatively unsaturated, acyl chains, or of more saturated, rigidifying acyl chain components. This feature of the invention is seen in Example 7, which examines blood/RES ratios in **liposomes** formed with PEG-PE, **cholesterol**, and **PC** having varying degrees of saturation (Table 4). As seen from the data in Table 5 in the example, high blood/RES ratios were achieved with in substantially all of the **liposome** formulations, independent of the extent of lipid unsaturation in the bulk **PC** phospholipid, and no systematic trend, as a function of degree of lipid saturation, was observed.

DETD . . . vesicle-forming lipids may be selected to achieve a selected degree of fluidity or rigidity, to control the stability of the **liposomes** in serum and the rate of release of entrapped drug from the **liposomes** in the bloodstream and/or tumor. The vesicle-forming lipids may also be selected, in lipid saturation characteristics, to achieve

desired **liposome** preparation properties. It is generally the case, for example, that more fluidic lipids are easier to formulate and down size. . .

DETD Similarly, it has been found that the percentage of **cholesterol** in the **liposomes** may be varied over a wide range without significant effect on observed blood/RES ratios The studies presented in Example 8A, with reference to Table 6 therein, show virtually no change in blood/RES ratios in the range of **cholesterol** between 0-30 mole percent.

DETD **Cholesterol**, or related **cholesterol** derivatives may be important, however, in regulating the rate of release of **liposome** entrapped therapeutic compounds into the bloodstream. The studies reported in Examples 15 and 16, for example, indicate that the rate of release of encapsulated polypeptide (peptide or protein) from **liposomes** in vitro (in the presence of human serum) or in vivo (in the bloodstream) is strongly dependent on **cholesterol** concentration PEG-**liposome** formulations containing high **cholesterol** (e.g., 30 mole percent or greater) release very little peptide or protein into serum in vitro, whereas decreasing amounts of **cholesterol** produce increasing loss of encapsulated polypeptide Similarly, and as described below, increased **cholesterol** in intravenously administered PEG-**liposomes** produced reduced release of encapsulated compound into the bloodstream (Example 16) and reduced physiological effect (Example 15). Thus, in accordance with one feature of the invention, the rate of release of compound from long-circulating **liposomes** can be controlled by the percent **cholesterol** included in the **liposomes**.

DETD . . . such as phosphatidylglycerol (PG). This can be seen from FIG. 7, which plots percent loss of encapsulated marker for PEG-PE **liposomes** containing either 4.7 mole percent PG (triangles) or 14 mole percent PG (circles). Virtually no difference in **liposome** retention in the bloodstream over a 24 hour period was observed

DETD In one embodiment, the **liposomes** are formulated to contain diglyceride at a mole ratio of up to 25 mole percent or more total **liposome** lipids Such **liposomes** are characterized by rapid **liposome** breakdown in the bloodstream, with release of encapsulated material, and the rate of breakdown can be selectively controlled by the percent of diglyceride included in the **liposomes**. The ability of the such **liposomes** to avoid uptake by the RES, and at the same time, to break down in the bloodstream over a period. . .

DETD B. Preparing the **Liposome** Composition

DETD The **liposomes** may be prepared by a variety of techniques, such as those detailed in Szoka et al, 1980. One method for preparing drug-containing **liposomes** is the reverse phase evaporation method described by Szoka et al and in U.S. Pat. No. 4,235,871. The reverse phase. . . or a few lipid bilayer shells. The method is detailed in Example 5A. This method is generally preferred for preparing **liposomes** with encapsulated proteins high encapsulation efficiencies (up to 50%) are possible, and thus protein loss or problems of recovery and. . .

DETD Multilamellar vesicles (MLVs) can be formed by simple lipid-film hydration techniques. In this procedure, a mixture of **liposome**-forming lipids of the type detailed above dissolved in a suitable solvent is evaporated in a vessel to form a thin. . .

DETD In accordance with one important aspect of the invention, the **liposomes** for intravenous injection are prepared to have substantially homogeneous sizes in a selected size range between about 0.1 and 0.4, and preferably 0.1 to 0.2 micron size ranges. **Liposomes** in this size range have sufficiently high encapsulation volumes for carrying therapeutically effective amounts of the compound to be administered. At lower **liposome** sizes, the ratio of **liposome**-encapsulated compound to free compound may be too low to achieve a requisite initial dose level of **liposome**-encapsulated compound in the bloodstream or may not remain in circulation due to extravasation. At the same time, 0.1-0.4 micron **liposomes** are small enough to give long blood circulation times, as discussed below, and also to allow sterilization by filtration.

DETD One effective sizing method for REVs and MLVs involves extruding an aqueous suspension of the **liposomes** through a polycarbonate membrane having a selected uniform pore size, typically 0.4, 0.2, and/or 0.1

largest sizes of the **liposomes** which are produced, particularly where the preparation is extruded two or more times through the same size membrane. This method of **liposome** sizing is used in preparing homogeneous-size REV and MLV compositions described in the examples below. A more recent method involves extrusion through an asymmetric ceramic filter. The method is detailed in U.S. Pat. No. 4,737,323 for **Liposome** Extrusion Method. Homogenization methods are also useful for down-sizing **liposomes**.

DETD It has also been discovered, in **liposome** processing studies carried out in support of the present invention, that **liposomes** coated with a hydrophilic polymer, such as PEG, can be formed with mean particle sizes of about 100-200 nm or. . . homogenization at less than 8,000 psi and at a temperature less than 50° C. This is in contrast to conventional **liposomes**, for which homogenization pressures of between 8,000-13,000 psi, and temperatures between about 50°-80° C. are required to produce particles in this size range (e.g., as reported in U.S. Pat. No. 4,753,788). In one method, reported in Example 5E, sized **liposomes** were prepared by homogenization at 8,000 psi at 50° C. at homogenization times up to 30 minutes, where the material. . .

DETD The **liposome** composition of the invention may also be prepared by diffusing a lipid derivatized with a hydrophilic polymer, such as PEG-**PC**, into preformed **liposomes**. In a typical method, **liposomes** prepared in the absence of polymer-derivatized lipid are incubated with micelles of the derivatized lipid, at a lipid concentration corresponding to the final mole percent of derivatized lipid which is desired. Thus, for example, to form PEG-**liposomes** containing 10 mole percent PEG, **liposomes** may be incubated with 10 mole percent PEG-PV micelles. Incubation is carried out with stirring until substantially all of the derivatized lipid has diffused into the **liposomes**. Typical incubation times are 2 hours at 60° C., or 24 hours at 37° C.

DETD The just-described method is useful, for example, when the **liposomes** are prepared to include covalently attached ligand surface molecules, such as antigen or antibody molecules, for **liposome** targeting or to achieve some other ligand-specific **liposome** interaction. Here, the **liposomes** are first reacted with the ligand, for covalent attachment to the **liposome** surface, according to conventional **liposome** coupling methods, and the ligand-coated **liposomes** are then mixed with the derivatized lipid, for anchoring hydrophilic polymer to the **liposome** surface.

DETD . . . (TNF-alpha, beta), and colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage); anticoagulants, such as TPA, prourokinase, and urokinase; **vaccines**, such as **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**; and other polypeptides (peptides and proteins), such as erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, insulin-like growth. . .

DETD As noted above, a polypeptide is preferably loaded passively by the reverse-phase emulsion method for preparing **liposomes**, although many other methods, such as solvent injection or lipid hydration may be employed. After **liposome** formation and sizing, free (unbound) drug can be removed by a variety of methods, for example, by gel filtration or. . .

DETD At the same time, the encapsulated compound is preferably present in an amount which, in a selected **liposome** dose, is between 3-20 times the amount of compound which would be given as a single therapeutic dose in free. . . administration. Thus, if the therapeutic dose of a peptide in free form is 1 μg for IV administration, a selected **liposome** dose will preferably contain between about 3-20 μg of the peptide. Since 10-20% of this compound, e.g., 2-4 μg, may be in non-encapsulated form, it will be appreciated that the total amount of **liposome** which can be administered may be limited by the maximum tolerated dose of the free compound It is clear that larger doses of **liposomes** can be administered by achieving higher ratios of

encapsulated as non-encapsulated compound. In general, this ratio is increased with larger **liposomes**, more complete free drug removal from the **liposome** composition, and greater **liposome** stability on storage.

DETD Such compounds may be encapsulated by passive loading, as above, during **liposome** formation by reverse evaporation phase, lipid hydration, solvent injection, or other **liposome** formation methods, and removed, after sizing by gel filtration or the like.

DETD Alternatively, drugs which form weak bases at physiological pH may be actively loaded into the **liposomes** at high drug concentration in the **liposomes**. One method for active loading drugs into **liposomes** is described in co-owned U.S. patent application Ser. No. 413,037, filed Sep. 28, 1988. In this method, **liposomes** are prepared in the presence of a relatively high ammonium ion, such as 0.125M ammonium sulfate. After sizing the **liposomes** to a desired size, the **liposome** suspension is treated to create an inside-to-outside ammonium ion gradient across the liposomal membranes. The gradient may be created by. . . with 0.15M NaCl or KCl, effectively replacing ammonium ions in the exterior phase with sodium or potassium ions. Alternatively, the **liposome** suspension may be diluted with a non-ammonium solution, thereby reducing the exterior-phase concentration of ammonium ions. The ammonium concentration inside the **liposomes** is preferably at least 10 times, and more preferably at least 100 to 1000 times that in the external **liposome** phase.

DETD The ammonium ion gradient across the **liposomes** in turn creates a pH gradient, as ammonia is released across the **liposome** membrane, and protons are trapped in the internal **liposome** phase. To load **liposomes** with the selected drug, a relatively dilute suspension of the **liposomes**, e.g., less than about 50 mM lipid, is mixed with an aqueous solution of the drug, and the mixture is. . . to equilibrate over a period of time, e.g., 24 hours at room temperature. In one typical method, a suspension of **liposomes** having a lipid concentration of 25 mg/ml is mixed with an equal volume of anthracycline drug at a concentration of. . .

DETD One of the requirements for extended compound release into the bloodstream, in accordance with the invention, is an extended **liposome** lifetime in the bloodstream with IV **liposome** administration. One measure of **liposome** lifetime in the bloodstream in the blood/RES ratio determined at a selected time after **liposome** administration, as discussed above. Blood/RES ratios for a variety of **liposome** compositions are given in Table 3 of Example 6. In the absence of PEG-derivatized lipids, blood/RES ratios were 0.03 or. . . ratio ranged from 0.2, for low-molecular weight PEG, to between 1.7-4 for several of the formulations, one of which lacks **cholesterol**, and three of which lack a charged phospholipid (e.g., PG).

DETD . . . 3. As noted in Section II above, the blood lifetime values are substantially independent of degree of saturation of the **liposome** lipids, presence of **cholesterol**, and presence of charged lipids.

DETD . . . best 24-hour blood/RES ratios which were reported in the above-noted patent was 0.9, for a formulation composed of GM_1, saturated **PC**, and **cholesterol**. The next best formulations gave 24-hour blood/RES values of about 0.5. Thus, typical 24-hour blood/RES ratios obtained in a number. . . to achieve high blood/RES with GM_1 or HPI lipids was dependent on the presence of predominantly saturated lipids in the **liposomes**.

DETD Plasma kinetics of a liposomal marker in the bloodstream can provide another measure of the enhanced **liposome** lifetime which is achieved by the **liposome** formulations of the present invention. FIGS. 7 and 8 discussed above show the slow loss of liposomal marker over a 24 hour period in typical PEG-**liposome** formulations, substantially independent of whether the marker is a lipid or an encapsulated water-soluble compound (FIG. 8). In both plots, the amount of liposomal marker present 24 hours after **liposome** injection is greater than 10% of the originally injected material.

DETD FIG. 9 shows the kinetics of **liposome** loss from the bloodstream for a typical PEG-**liposome** formulation and the same **liposomes** in the absence of a PEG-derivatized lipid. After 24 hours, the percent marker

remaining in the PEG-**liposomes** was greater than about 20%, whereas the conventional **liposomes** showed less than 5% retention in the blood after 3 hours, and virtually no detectable marker at 24 hours.

DETD The results seen in FIGS. 7-9 are consistent with 24 hour blood **liposome** values measured for a variety of **liposome** formulations, and reported in Tables 3 and 5-7 in Example 6-9 below. As seen Table 3 in Example 6, the percent dose remaining at 24 hours was less than 1% for conventional **liposomes**, versus at least 5% for the PEG-**liposomes**. In the best formulations, values between about 20-40% were obtained. Similarly in Table 5 from Example 7, **liposome** levels in the blood after 24 hours (again neglecting two low recovery values) were between 12 and about 25 percent. . .

DETD The effect of **liposome** size on blood lifetime was been investigated by comparing loss of liposomal marker in intravenously injected **liposomes** having selected sizes between about 0.1 and 0.25 microns. Experimental details are given in Example 10. The results, given in FIG. 10, show about 10% or greater **liposome** marker present in the blood after 24 hours for each of the **liposome** formulations. Highest blood lifetimes were achieved with the smallest **liposomes**. Thus, although all of the **liposome** preparations give high blood lifetimes, it is also clear that **liposome** size can be selected to produce desired increase or decrease in total drug release time.

DETD The enhancement in **liposome** blood circulation time achieved with two other biocompatible hydrophilic polymers, polylactic acid and polyglycolic acid, is seen in FIG. 11, which shows loss of **liposome** marker during the 24-hour period after intravenous **liposome** injection. The percent marker remaining at 24 hours is about 3.9 percent for polylactic acid (solid squares), and about 6% for polyglycolic acid (open triangles) These values compare with the 0.1-1% retention seen in conventional **liposomes** after 24 hours.

DETD The data relating to both blood/RES ratios and to **liposome** retention time in the bloodstream which were obtained from an model animal system can be reasonably extrapolated to humans and veterinary animals of interest. This is because uptake of **liposomes** by liver and spleen has been found to occur at similar rates in several mammalian species, including mouse, rat monkey,. . . Juliano; Richardson; Lopez-Berestein). This result likely reflects the fact that the biochemical factors which appear to be most important in **liposome** uptake by the RES--including opsinization by serum lipoproteins, size-dependent uptake effects, and cell shielding by surface moieties--are common features of. . .

DETD In addition to long circulating halflives, another important property of the **liposomes** of the present invention is the ability to release entrapped compound, at a therapeutically effective dose rate in the bloodstream.

DETD As discussed above, the **liposome** size between 0.1 and 0.4 microns allows relatively high compound loading in the **liposomes**, for effective compound release in the bloodstream even at relatively low **liposome** concentrations. This is typically an important consideration since the total quantity of **liposomes** which can be administered will be limited, due to the unavoidable presence of some free compound in an injected preparation.

DETD Another consideration is the ability of an entrapped compound to be released from the **liposomes** during circulation in the bloodstream. This feature is illustrated in the studies described in Example 15 below. Here PEG-**liposomes** containing entrapped vasopressin (a 1 kilodalton peptide) were prepared with increasing concentrations of **cholesterol**, from 0 to about 30 mole percent. In vitro measurements of peptide release from the **liposomes** in serum indicated that substantially less peptide is released with greater amounts of **cholesterol**. The rate of release of the peptide hormone in vivo was determined by its diuretic effect, as measured by decreased urine output, in a period 1-8 days following intravenous administration of the **liposomes**. Details of the study are given in Example 15. As seen in FIG. 14, the short-term effect on urine flow was dependent on **cholesterol** content, the PEG-**liposomes** with highest **cholesterol**

producing the greatest hormone effect. Similarly, in the period 2-8 days following IV administration of the **liposomes**, the two PEG-**liposome** formulations having the lowest **cholesterol** concentration gave the greatest hormone effect, indicating higher release rates from the **liposomes**.

DETD The study reported in Example 16 demonstrates a similar ability to control release rates of large proteins from long-circulating **liposomes**. Here PEG-**liposomes** containing encapsulated M-CSF (a 55 kilodalton protein) were examined for percent retention in the bloodstream, of both lipid and protein components. The data plotted in FIG. 15 show **liposome** lipid (solid triangles) and protein (solid circles) kinetics during a 24-hour period after IV injection for a PEG-lipsome formulation containing 30 mole percent **cholesterol**. The data indicate about 20% loss of encapsulated material over 24 hours, as discussed in Example 16.

DETD FIG. 16 shows a similar plot for PEG-**liposomes** without **cholesterol**. The data here show a 40-50% loss of M-CSF after 24 hours. When lipid and protein markers are normalized at the first time point (15 min) over the plots of relative protein release from **cholesterol** (solid circles) and no-**cholesterol** (solid triangles) PEG-**liposomes** are obtained. The plots illustrate the markedly different protein release kinetics which can be obtained with long circulating **liposomes**, by varying **cholesterol** content of the **liposomes**. Another feature of the data, shown in FIG. 17, is the relatively high percent (at least 3%) of initially injected. . .

DETD IV. Intravenous **Liposome** Treatment

DETD In practicing the method, there is provided a **liposome** composition such as described above, containing the compound in **liposome**-entrapped form. The size, lipid composition, and extent of compound loading of the drug are selected, according to the desired release. . .

DETD . . . single dose. Thus, for example, if a therapeutically effective single dose of a compound is 10 μg/Kg body weight, the **liposome** composition would be injected at a **liposome** dose of at least 30 μg compound/Kg, and typically between 50-200 μg compound/Kg body weight. As noted above, the amount. . . for example, if the maximum tolerated dose of a compound in free form is 2 μg/Kg body weight, and the **liposome** composition contains 10% non-entrapped compound, the highest dose of **liposome** composition which can be given is 20 μg/Kg body weight.

DETD Studies on the treatment of L1210 leukemia in mice with cytosine arabinoside (araC), in free form and entrapped in PEG-**liposomes** indicates that the **liposome** composition produced about a 250-300% increase in survival time when administered in PEG-liposomal form, compared with about a 120% increase. . .

DETD FIG. 13 shows the effect on urine flow by vasopressin administered IV in PEG-**liposomes**, at dosage levels of 2 μg (solid squares), 8 μg (solid triangles) and 24 μg (solid circles). A pronounced reduction. . . with a slight rebound toward control values by day 2--an effect likely due, in part, to free peptide in the **liposome** formulation. In addition, a marked reduction in urine flow was observed for at least 7 days following IV administration. It. . . g dose, with no further effect observed at 24 μg. The same long-term therapeutic effect of vasopressin was observed in PEG-**liposomes** containing various amounts of **cholesterol**, as seen in FIG. 14, and as discussed above. The data in this figure also illustrate increased short-term (1-2 days) and decreased long-term (2-8 days) effect seen with low **cholesterol** **liposomes** having the highest peptide release rates.

DETD . . . an extended period and (b) the rate of release of the protein into the bloodstream can be selectively controlled by **liposome** composition.

DETD V. Subcutaneous **Liposome** Treatment

DETD In accordance with another aspect of the invention, it has been discovered that the long-live **liposome** composition of the invention is also effective for slow release of a **liposome**-entrapped compound from a subcutaneous (SubQ) site into the bloodstream. In particular, it has

been observed and therapeutic effects of up to 3 weeks or more can be achieved by a single subcutaneous injection of the **liposome** composition of the invention.

DETD . . . treatment method involving SubQ injection of free vasopressin was compressed, for duration of physiological effect, with vasopressin administered SubQ in PEG-**liposomes**. FIG. 18A shows the depression in urine production observed with SubQ administration of free vasopressin at doses of 2 μg. . .

DETD . . . of effective activity of an therapeutic compound which can be administered intravenously in a therapeutically effective amount. The method utilizes **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with a hydrophilic polymer, and. . . having a selected mean particle diameter in the size range preferably between about 0.07-0.15 microns, and having the compound in **liposome**-entrapped form.

DETD The **liposome** composition is administered subcutaneously at a dose of the composition which contains an amount of the **liposome**-entrapped compound which is at least ten times the therapeutically effective intravenously administered amount.

DETD The ability of the **liposome** composition of the invention to produce a long-term therapeutic effect from a SubQ site suggests that the **liposomes** taken up from this site through the lymphatics may be able to successfully evade the normal lymphatic clearance mechanisms, including dendritic cells. This mechanism is supported by studies on araC administration by the interperitoneal (IP) route by PEG-**liposomes**. Briefly, it was found that PEG-**liposomes** containing entrapped araC and administered by IP route produced a 250-300% increase in survival in animals having L1210 leukemias, compared with about 120% increase in survival with the free drug given by the IP route. The results suggests that PEG-**liposomes** are capable of migrating from the peritoneum through the lymphatics into the bloodstream.

DETD **Cholesterol** (Chol) was obtained from Sigma (St. Louis, Mo.). Sphingomyelin (SM), egg **phosphatidylcholine** (lecithin or **PC**), partially hydrogenated **PC** having the composition IV40, IV30, IV20, IV10, and IV1, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), dipalmitoyl-phosphatidyl glycerol (DPPG), dipalmitoyl **PC** (DPPC), dioleyl **PC** (DOPC) and distearoyl **PC** (DSPC) were obtained from Avanti Polar Lipids (Birmingham, Ala.) or Austin Chemical Co (Chicago, Ill.).

DETD . . . been reduced to about three to four ethylene oxide residues. The product prepared was used for a preparation of PEG-PE **liposomes**.

DETD . . . was emulsified by sonication for 1 minute, maintaining the temperature of the solution at or below room temperature. Where the **liposomes** were prepared to contain encapsulated [^{125} I] tyraminyl-inulin, such was included in the phosphate buffer at a concentration of about. . .

DETD The **liposomes** were extruded twice through a polycarbonate filter (Szoke, 1978), having a selected pore size of 0.4 microns or 0.2 microns. **Liposomes** extruded through the 0.4 micron filter averaged 0.17+ (0.15) micron diameters, and through the 0.2 micron filter, 0.16 (0.05) micron diameters. Non-encapsulated [^{125} I] tyraminyl-inulin was removed by passing the extruded **liposomes** through Sephadex G-50 (Pharmacia).

DETD Multilamellar vesicle (MLV) **liposomes** were prepared according to standard procedures by dissolving a mixture of lipids in an organic solvent containing primarily $CHCl_3$ and. . .

DETD The size of the **liposome** samples was controlled by extrusion through defined pore polycarbonate filters using pressurized nitrogen gas. In one procedure, the **liposomes** were extruded one time through a filter with pores of 0.4 μm and then ten times through a filter with pores of 0.1 μm. In another procedure, the **liposomes** were extruded three times through a filter with 0.2 m pores followed by repeated extrusion with 0.05 μm pores until. . . determined by DLS. Unencapsulated aqueous components were removed by passing the extruded sample through a gel permeation column separating the **liposomes** in the void volume from the small molecules in the included volume.

DETD C. Loading Ga^{67} into DF containing **liposomes**

DETD The protocol for preparation of Ga^{67} -DF labeled **liposomes** as adapted from known procedures (Gabizon). Briefly, **liposomes** were prepared with the ion chelator desferal mesylate encapsulated in the internal aqueous phase to bind irreversibly Ga transported through. . .

DETD **Liposome** particle size distribution measurements were obtained by DLS using a NICOMP Model 200 with a Brookhaven Instruments BI-2030AT autocorrelator attached.. . .

DETD . . . molecular weight 1900 and DSPE was prepared as in Example 2. The PEG-PE lipid was combined with and hydrogenated soy **PC** (HSPC) and **cholesterol** in a mole ratio of 5:55:40. A total of 60 mg of lipid were hydrated in aqueous buffer solution containing. . . 19B, for particle size measurements by the Nicomp and Coulter Analysers, respectively. As seen, particle size reduction stabilized at about 100-**150 nm** after 3-5 minutes. Under the homogenization conditions employed, the material was cycled through the homogenizer about one time per minute.

DETD **Liposome** Blood Lifetime Measurements

DETD In vivo studies of **liposomes** were performed in two different animal models: Swiss-Webster mice at 25 g each and laboratory rats at 200-300 g each. The studies in mice involved tail vein injection of **liposome** samples at 1 μM phospholipid/mouse followed by animal sacrifice after a defined time and tissue removal for label quantitation by. . . establishment of a chronic catheter in a femoral vein for removal of blood samples at defined times after injection of **liposome** samples in a catheter in the other femoral artery at 3-4M phospholipid/rat. The percent of the injected dose remaining in. . .

DETD B. Time Course of **Liposome** Retention in the Bloodstream

DETD . . . 1900 and 1-palmitoyl-2-oleyl-PE (POPE) was prepared as in Example 2. The PEG-POPE lipid was combined with and partially hydrogenated egg **PC** (PHEPC) in a lipid:lipid mole ratio of about 0.1:2, and the lipid mixture was hydrated and extruded through a 0.1. .

DETD The **liposome** composition was injected and the percent initial injected dose in mice was determined as described in Example 4, at 1,. . .

DETD C. 24 Hour Blood **Liposome** Levels

DETD Studies to determine percent injected dose in the blood, and blood/RES ratios of a liposomal marker, 24 hours after intravenous **liposome** injection, were carried out as described above. **Liposome** formulations having the compositions shown at the left in Table 3 below were prepared as described above. Unless otherwise noted, the lipid-derivatized PEG was PEG-1900, and the **liposome** size was 0.1 micron. The percent dose remaining in the blood 24 hours after intravenous administration, and 24-hour blood/RES ratios. . .

DETD TABLE 3

Lipid Composition*	24 Hours After IV Dose % Injected Dose in Blood	B/RES
PG:**PC**:Chol (.75:9.25:5)	0.2	0.01
PC:Chol (10:5)	0.8	0.03
PEG-DSPE:**PC**:Chol	23.0	3.0
PEG-DSPE:**PC**:Chol (250 nm)	9.0	0.5
PEG_{5000} -DSPE:**PC**:Chol	21.0	2.2
PEG_{750} -DSPE:**PC**:Chol	3.2	0.33
PEG:$_{120}$ -DSPE:**PC**:Chol	5.0	0.2
PEG-DSPE:**PC** (0.75:9.25)	22.0	1.7
PEG-DSPE:PG:**PC**:Chol		

(0.75:2.25:7:5)
PEG-DSPE:NaCholSO$_4$:**PC**:Chol
25.0 2.5
(0.75:0.75:9.25:4.25)

*All formulations contain 33% **cholesterol** and 7.5% charged component and were 100 nm mean diameter except as noted. PEGDSPE consisted of PEG_{1900} except as noted.. . .

DETD As seen, percent dose remaining in the blood 24 hours after injection ranged between 5-40% for **liposomes** containing PEG-derivatized lipids. By contrast, in both **liposome** formulations lacking PEG-derivatized lipids, less than 1% of **liposome** marker remained after 24 hours. Also as seen in Table 3, blood/RES ratios increased from 0.01-0.03 in control **liposomes** to at least 0.2, and as high as 4.0 in **liposomes** containing PEG-derivatized **liposomes**.

DETD Effect of Phospholipid Acl-Chain Saturation on Blood/RES Ratios in PEG-PE **Liposomes**

DETD . . . prepared as in Example 2. The PEG-PE lipids were formulated with selected lipids from among sphingomyelin (SM), fully hydrogenated soy **PC** (**PC**), **cholesterol** (Chol), partially hydrogenated soy **PC** (PHSPC), and partially hydrogenated **PC** lipids identified as **PC** IV1, IV10, IV20, IV30, and IV40 in Table 4. The lipid components were mixed in the molar ratios shown at. . .

DETD TABLE 4

Phase Transition Egg **PC** Temperature Range Form	Mole % Fatty Acid Comp. °13 C. 18:0	18:1	18:2	20:0	20:1-4	22:0	22:1-6
Native	<0	12	30	15.	.	.	

DETD TABLE 5

	Blood RES	B/RES	% Remaining
PEG-PE:SM:**PC**:Chol	19.23	6.58	2.92 49.23
0.2:1:1:1			
PEG-PE:PHSPC:Chol	20.54	7.17	2.86 55.14
0.15:1.85:1			
PEG-PE:**PC** IV1:Chol	17.24	13.71	1.26 60.44
0.15:1.85:1			
PEG-PE:**PC** IV1:Chol	19.16	10.07	1.90 61.87
(two animals)			
0.15:1.85:1			
PEG-PE:**PC** IV10:Chol	12.19	7.31	1.67 40.73
(two animals)			
0.15:1.85:1			
PEG-PE:**PC** IV10:Chol	2.4	3.5	0.69 12.85
0.15:1.85:1			
PEG-PE:**PC** IV20:Chol			

0.15:1.85:1
PEG-PE:**PC** IV20:Chol
5.2 5.7 0.91 22.1
0.15:1.85:1
PEG-PE:**PC** IV40:Chol
19.44 8.87 2.19 53.88
0.15:1.85:1
PEG-PE:**PC** IV:Chol
20.3 8.8 2.31 45.5
0.15:1.85:0.5
PEG-PE:EPC:Chol
15.3 9.6 1.59 45.9
0.15:1.85:1

DETD . . . that the blood/RES ratios are largely independent of the fluidity, or degree of saturation of the phospholipid components forming the **liposomes**. In particular, there was no systematic change in blood/RES ratio observed among **liposomes** containing largely saturated **PC** components (e.g., IV1 and IV10 **PC**'s), largely unsaturated **PC** components (IV40), and intermediate-saturation components (e.g., IV20).

DETD . . . 5) indicates that the degree of saturation of the derivatized lipid is itself not critical to the ability of the **liposomes** to evade uptake by the RES.

DETD Effect of **Cholesterol** and Ethoxylated **Cholesterol** on Blood/RES Ratios in PEG-PE **Liposomes**

DETD A. Effect of added **cholesterol**

DETD . . . as described in Example 2. The PEG-PE lipids were formulated with selected lipids from among sphingomyelin (SM), fully hydrogenated soy **PC** (**PC**), and **cholesterol** (Chol), as indicated in the column at the left in Table 6 below. The three formulations shown in the table contain about 30, 15, and 0 mole percent **cholesterol**. Both REV's (0.3 micron size) and MLV's (0.1 micron size) were prepared, substantially as in Example 4, with encapsulated tritium-labeled. . .

DETD . . . blood 2 and 24 hours after administration, given at the right in Table 6 below, show no measurable effect of **cholesterol**, in the range 0-30 mole percent.

DETD . . . In Blood

	2 HR.	24 HR.	2 HR.	24 HR.
	^{3}H Aqueous			
			^{14}C-	
^{3}H-Inulin Label (Leakage)				
			Lipid Label	

---

1) SM:**PC**:Chol:PEG-DSPE 1:1:1:0.2				
100 nm MLV	19	5	48	24
300 nm REV	23	15	67	20
2) SM:**PC**:Chol:PEG-DSPE 1:1:0.5:0.2				
300 nm REV	23	15	71	17
3) SM:**PC**:PEG-DSPE 1:1:0.2				
100 nm MLV	19	6	58	24
300 nm REV	32	23	76	43

DETD B. Effect of ethoxylated **cholesterol**

DETD Methoxy-ethoxy-**cholesterol** was prepared by coupling methoxy ethanol to **cholesterol** via the trifluorosulfonate coupling method described in Section I. PEG-PE composed of methoxy PEG, molecular weight 1900 and was derivatized. . . DSPE as described in Example 2. The PEG-PE lipids were formulated with selected lipids from among distearylPC (DSPC), hydrogenated soy **PC** (HSPC), **cholesterol**, and ethoxylated **cholesterol**, as indicated at the left in Table 7. The data show that (a) ethoxylated **cholesterol**, in combination with PEG-PE, gives about the same degree of enhancement of **liposome** lifetime in the blood as

moderate degree of enhancement of **liposome** lifetime, but substantially less than that provided by PEG-PE.

DETD Effect of Charged Lipid Components on Blood/RES Ratios in PEG-PE **Liposomes**

DETD . . . described in Example 2. The PEG-PE lipids were formulated with lipids selected from among egg PG (PG), partially hydrogenated egg **PC** (PHEPC), and **cholesterol** (Chol), as indicated in the FIG. 7. The two formulations shown in the figure contained about 4.7 mole percent (triangles). . .

DETD The percent of injected **liposome** dose present 0.25, 1, 2, 4, and 24 hours after injection re plotted for both formulations in FIG. 7. As seen, the percent PG in the composition had little or no effect on **liposome** retention in the bloodstream. The rate of loss of encapsulated marker seen is also similar to that observed for similarly prepared **liposomes** containing no PG.

DETD Effect of **Liposome** Size on Blood Lifetime

DETD PEG-DSPE, prepared as above with PEG-1900, was formulated with partially hydrogenated egg **PC** (PHEPC), and **cholesterol** (Chol), at a mole ratio of 0.15: 1.85: 1. The **liposomes** were sized by extrusion through 0.25, 0.15 or 0.1 micron polycarbonate filters, to produce **liposome** sizes of about 0.4, 0.2, and 0.1 microns, respectively. Non-encapsulated ^{3}H-inulin was removed by gel filtration.

DETD Each of the three **liposome** were injected intravenously, and the percent of injected **liposome** marker in the blood was measured at 1, 2, 3, 4, and 24 hours, with the results shown in FIG. 10. All three formulations show long blood half-lives, as evidence by at least bout 10% **liposome** marker remaining after 24 hours. The 0.1 micron formulation (solid squares) is longer lived than the 0.2 micron formulation (solid. . .

DETD Polylactic acid-DSPE or polyglycolic acid-DSPE, prepared as above, was formulated with lipids selected from among hydrogenated soy **PC** (HSPC), and **cholesterol** (Chol), at a weight ratio of either 2:3.5:1 or 1:3.5:1. The **liposomes** were sized by extrusion through a 0.1 micron polycarbonate filter. The **liposomes** were labeled by ^{67}Ga-DF as described in Example 5.

DETD These **liposome** formulations were injected intravenously, and the percent of injected **liposome** marker in the blood was measured at 1, 2, 3, 4, and 24 hours, with the results shown in FIG. 11. When normalized at 15 minutes, about 3.9% of the **liposome** marker was present after 24 hours with polylactic acid-DSPE (solid squares). An average value of about 6% of the **liposome** marker was present after 24 hours after with polyglycolic acid-DSPE (open triangles). This compares with less than 1% remaining for similarly prepared **liposomes** without the hydrophilic polymers.

DETD Plasma Kinetics of PEG-Coated and Uncoated **Liposomes**

DETD . . . weight 1900 and distearyl PE (DSPE) was prepared as in Example 2. The PEG-PE lipids were formulated with PHEPC, and **cholesterol**, in a mole ratio of 0.15:1.85:1. A second lipid mixture contained the same lipids, but without PEG-PE. **Liposomes** were prepared from the two lipid mixtures as described in Example 5, by lipid hydration in the presence of DF. . .

DETD The two **liposome** compositions (0.4 ml) were injected IV in animals, as described in Example 6. At time 0.25, 1, 3 or 5,. . . a percentage of the amount measured immediately after injection. The results are shown in FIG. 9. As seen, the PEG-coated **liposomes** have a blood halflife of about 11 hours, and nearly 30% of the injected material is present in the blood after 24 hours. By contrast, uncoated **liposomes** showed a halflife in the blood of less than 1 hour. At 24 hours, the amount of injected material was. . .

DETD Large **unilamellar liposomes** were prepared according to the reverse phase evaporation procedure (Szoka and Papahadjopoulos, 1978). The lipid composition used in preparing the **liposomes** was PEG-DSPE, **PC**, sphingomyelin, and **cholesterol**, in a mole ratio 0.2:1:1:1. Lipid mixtures for a 1 ml final volume of **liposomes** with a phospholipid concentration of 10 μmol/ml were dissolved in chloroform and

evaporated to dryness under reduced pressure. After residual . . . additional drug-free buffer added to give a 1 ml solution immediately after gel dispersion. The vasopressin content of the resulting **liposomes** was determined by separating free from **liposome**-bound by gel filtration on Bio-Gel A15.

DETD The **liposomes** were extruded through 0.4 μm Nuclepore filters (Olsen et al., 1979) and particle size distribution measured by dynamic light scattering. . .

DETD PEG-**liposome** preparations from above were administered intravenously to rats prepared as in Example 13, at liposomal doses 2 μg (solid squares). . . dose-dependent depression in urine production in the first day after drug administration, presumably resulting predominantly from non-entrapped vasopressin in the **liposome** formulations. In contrast to free peptide administration, however, all three formulation produced a significant inhibition in urine production with respect to control (open circles) over a 2-8 day period after **liposome** administration.

DETD Treatment with PEG-Liposomal Vasopressin: Effect of **Liposome Cholesterol** Concentration

DETD Large **unilamellar liposomes** were prepared as in Example 14, with **liposomes** containing either 33, 16, or 0 mole percent **cholesterol**. Vasopressin encapsulation, **liposome** sizing, and free peptide removal was carried out as in Example 14.

DETD The three PEG-**liposome** preparations were administered intravenously to rats prepared as in Example 13, at liposomal doses giving 8 μg vasopressin, and the percent predosage urine production was measured over a 9 day period following **liposome** administration. The results are shown in FIG. 14, for saline control (open circles) and PEG-**liposomes** with 0 (solid squares), 16 (solid triangles), and 33 (solid circles) mole percent **cholesterol**.

DETD The day-1 response shows a marked dependence on percent **cholesterol**, with the greatest effect on urine flow being produced in **liposomes** with the lowest mole ratio of **cholesterol**. This result is consistent with in vitro stability studies of vasopressin release from PEG-**liposomes** in serum: In the presence of serum, little release of vasopressin was seen in **liposomes** containing greater than 30 mole percent **cholesterol**. By contrast, formulations containing reduced amounts of **cholesterol** showed increasingly higher release rates of encapsulated peptide. Thus, it would appear that the significantly higher diuretic effect seen after 1 day with low and no **cholesterol** formulations is due to the presence of free peptide released from the **liposomes** in serum.

DETD Interestingly, all three formulations produced a marked, and substantially similar diuretic effect over a 2-8 day period following **liposome** drug administration, as was seen in the method described in Example 14.

DETD Blood Clearance Kinetics of M-CSF from PEG-**Liposomes**

DETD A lipid film containing PEG-DSPE, PHEPC IV-40, **cholesterol**, and α-tocopherol, in a mole ratio 5:61:33:1, was hydrated with distilled water, and the resulting MLVs were sonicated for 30. . .

DETD . . . an acetone/dry bath, and lyophilized overnight. The dried material was rehydrated in 0.8 ml of distilled water, and the resulting **liposome** suspension was extruded 1 time through a 0.4μ polycarbonate filter, and 3 times through a 0.2μ filter. The sized **liposomes** were diluted to 10 ml in distilled water, washed washed two times with high speed centrifugation, and the washed pellet. . .

DETD PEG-**liposome** preparations from above were administered intravenously to animals as in Example 6, and the blood levels of M-CSF were measured. . . 14. Similar measurements were made for an equivalent amount of M-CSF administered in solution form. The plasma kinetics for the PEG-**liposome** formulation containing 30 mole percent **cholesterol** are shown in FIG. 15. The data show rapid clearance of free protein (solid triangles) with less than 1% protein remaining in the blood at 24 hours, compared with about 8% for **liposome**-associated protein (solid circles). Percent **liposomes** remaining in the bloodstream, as judged by

percent lipid marker, was slightly greater than 100%. A comparison of the clearance. . . of rates of the lipid and protein markers indicates that about 20% of the protein marker was released from the **liposomes** by 24 hours post injection.

DETD The plasma kinetics obtained with **cholesterol**-free PEG-**liposomes** is shown in FIG. 16. Percent **liposomes** remaining after 24 hours was about 8.5 (solid triangles) compared with about 4.5% for **liposome**-associated M-CSF. The results indicated that about 40-50% of the originally encapsulated protein leaked from the **liposomes** in the 24-hour period post injection.

DETD The radioactive counts in the **liposome** lipid marker and in the encapsulated protein were normalized to 100% initial values, and the percent injected dose released into the bloodstream over time was then determined from the difference between the normalized protein and normalized **liposome** marker radioactivity levels. A plot of the calculated values of percent protein released at 1, 2, 4, and 24 hours. . .

DETD The plot for the **cholesterol**-free formulation (solid triangles) shows a protein release peak at 2 hours, with a gradual decline in amount released in the 2-24 hour period in the no-**cholesterol** formulation (solid triangles). The amount of protein released from the **liposomes** at 24 hours was between 3-4 percent of the total administered.

DETD The plot for the formulation containing 30 mole percent **cholesterol** (solid circles) shows a gradual increase in release protein release rate over 24 hours. The amount of protein released from the **liposomes** at 24 hours was about 3 percent of the total administered. Thus, both formulations showed relatively high levels of protein. . .

DETD Subcutaneously Administered **Liposomes**

DETD . . . prepared by thin-film hydration as described in Example 5. The lipid composition of the thin film was PEG-DSPE, HEPC, and **cholesterol**, in a mole ratio 0.15:1.85:1. The thin film was hydrated with an aqueous buffer (5 mM Tris, 100 mM NaCl,. . . micron polycarbonate membrane, and free (non-encapsulated) peptide was removed by gel filtration, as in Example 15. The final concentration of PEG-**liposomes** in the suspension was 100 μM/ml.

DETD Vasopressin encapsulated in PEG-**liposomes**, prepared as above, was administered subcutaneously (1 ml) to animals as above. The doses administered were 25 μg (solid triangles),. . .

DETD Although the invention has been described and illustrated with respect to specific **liposome** formulations, **liposome**-entrapped compounds, and treatment methods, it will be apparent that a variety of related compositions, compounds and treatment methods without departing. . .

1. A **liposome** composition effective to extend, to at least 24 hours, the period of effective activity of a therapeutic compound which can. . . and which is cleared in free form from the bloodstream with a halflife of less than about 4 hours, comprising **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivatized with a polymer selected from. . . having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound in **liposome**-entrapped form, for intravenous administration at a dose of the composition which contains an amount of the compound in **liposome**-entrapped form which is at least three times such therapeutically effective amount.

5. A **liposome** composition effective to extend, to at least 48 hours, the period of therapeutic activity of a polypeptide which can be. . . halflife of less than about 4 hours, and whose therapeutically active blood concentration is in the picogram-nanogram/ml concentration range, comprising **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with a polymer selected from. . . having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the polypeptide in **liposome**-entrapped form, for intravenous administration at a dose of the composition which contains an amount of the polypeptide **liposome**-entrapped form which is at least three times such therapeutically effective amount.

. . . the polypeptide is a peptide hormone which is therapeutically active at a plasma concentration in the picogram/ml range, and the **liposome** composition is effective to release the hormone in a therapeutically effective dose for a period of at least five days. . .

. . . (1,2,3,4,5,6,7), tissue necroses factor (TNF-alpha, beta), colony stimulating factors (-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha,. . .

. . . and which has a halflife in the bloodstream in free form of less than about 4 hours, comprising providing a **liposome** composition containing **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with ah polymer selected from. . . particle diameter in the size range between about 0.1 to 0.4 microns, and the compound at least about 70% in **liposome**-entrapped form, and administering the **liposome** composition intravenously to a subject at a dose which contains an amount of the compound which is at least three. . .

. . . necroses factor (TNF - alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha,. . .

18. A **liposome** composition effective to extend, to at least one week, the period of effective activity of a therapeutic compound which can be administered in a therapeutically effective amount, comprising **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with a polymer selected from. . . having a selected mean particle diameter in the size range between about 0.1 to 0.4 microns, and the compound in **liposome**-entrapped form, for subcutaneous administration at a dose of the composition which contains an amount of the compound in **liposome**-entrapped form which is at least ten times such therapeutically effective intravenously administered amount.

. . . necrosis factor (TNF - alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha,. . .

. . . period of effective activity of a therapeutic compound which can be administered in a therapeutically effective amount, comprising providing a **liposome** composition containing **liposomes** (i) composed of vesicle-forming lipids and between 1-20 mole percent of a vesicle-forming lipid derivitized with a polymer selected from. . . particle diameter in the size range between about 0.1 to 0.4 microns, and the compound at least about 70% in **liposome**-entrapped form, and administering the composition subcutaneously to a subject at a dose which contains an amount of the compound in **liposome**-entrapped form which is at least ten times such therapeutically effective intravenously administered amount.

. . . (1,2,3,4,5,6,7), tissue necroses factor (TNF-alpha, beta), colony stimulating factors (M-CSF (macrophage), G-CSF (granulocyte), GM-CSF (granulocyte, macrophage), TPA, prourokinase, and urokinase, **HIV**-1 **vaccine**, hepatitis B **vaccine**, malaria **vaccine**, and melanoma **vaccine**, erythropoietin (EPO), factor VIII, bone growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, tumor growth factors (alpha,. . .

24. A **liposome** composition composed of vesicle-forming lipids and a

vesicle forming lipid derivatized with a hydrophilic polymer selected from the group consisting of. . .

=> d his

(FILE 'HOME' ENTERED AT 09:25:07 ON 12 JUL 2004)

FILE 'USPATFULL' ENTERED AT 09:25:18 ON 12 JUL 2004

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L1         33450 S (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L2          3069 S L1 AND (ALUMINUM HYDROXIDE)
L3          1785 S L1 AND (UNILAMELLAR)
L4          1772 S L3 AND LIPOSOME?
L5           434 S L4 AND (PHOSPHATIDYLCHOLINE)
L6           402 S L5 AND CHOLESTEROL
L7           107 S L6 AND MUCOSAL
L8            83 S L7 AND ORAL
L9            22 S L8 AND AY<2000
L10        13577 S L1 AND LIPOSOME?
L11          897 S L10 AND LIPOSOME?/CLM
L12          244 S L11 AND UNILAMELLAR
L13           18 S L12 AND UNILAMELLAR/CLM
L14           17 S L13 AND (CHOLESTEROL)
L15           17 S L14 AND (PHOSPHATIDYLCHOLINE OR PHOSPHATIDYL CHOLINE OR PC)
L16            2 S L15 AND IMMUNOGENIC/CLM
L17           15 S L15 NOT L16
                E FRIEDE MARTIN/IN
L18           11 S E3
L19            3 S L18 AND AY<2000
L20        37491 S (VACCIN? OR IMMUNOGENIC COMPOSITION?)
L21        17084 S L20 AND LIPOSOME?
L22         1926 S L21 AND UNILAMELLAR
L23         1153 S L22 AND (PHOSPHATIDYLCHOLINE OR PHOSPHATIDYL CHOLINE OR PC)
L24         1063 S L23 AND CHOLESTEROL
L25           53 S L24 AND (70 NM OR 150 NM)
L26           34 S L25 AND (HIV OR HUMAN IMMUNODEFICIENCY VIRUS)
L27            8 S L26 AND AY<2000
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=> e thibodeau lise/in

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E1            2     THIBODEAU LESLIE N/IN
E2            1     THIBODEAU LESLIE NEIL/IN
E3            0 --> THIBODEAU LISE/IN
E4            4     THIBODEAU MARK JASON/IN
E5            1     THIBODEAU MARVIN J/IN
E6            2     THIBODEAU MICHAEL J/IN
E7            1     THIBODEAU PAUL H/IN
E8            1     THIBODEAU PIERRE/IN
E9            1     THIBODEAU RAYMOND J/IN
E10           1     THIBODEAU RAYMOND JON/IN
E11           1     THIBODEAU RENE J/IN
E12           1     THIBODEAU RHEAL A/IN
```

=> e lavallee claude/in

```
E1            1     LAVALLEE BRADLEY C/IN
E2            1     LAVALLEE CAROLYN/IN
E3            5 --> LAVALLEE CLAUDE/IN
E4            7     LAVALLEE DAVID A/IN
E5            2     LAVALLEE DAVID ANTHONY/IN
E6            1     LAVALLEE DAVID G/IN
E7            2     LAVALLEE DAVID K/IN
E8            2     LAVALLEE DONALD C/IN
E9            6     LAVALLEE ERIC/IN
E10           1     LAVALLEE FRAN CEDILLA OIS A/IN
E11          25     LAVALLEE FRANCOIS A/IN
E12           4     LAVALLEE GERALD A/IN
```

L28 5 "LAVALLEE CLAUDE"/IN

=> d l28,cbib,1-5

L28 ANSWER 1 OF 5 USPATFULL on STN
2003:312844 Extrudable thermoplastic compositions.
Dillon, Maria P., St. Paul, MN, UNITED STATES
Horns, John H., Edina, MN, UNITED STATES
Lavallee, Claude, London, CANADA
3M Innovative Properties Company (U.S. corporation)
US 2003220450 A1 20031127
APPLICATION: US 2002-151788 A1 20020520 (10)
DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 2 OF 5 USPATFULL on STN
2001:136722 Polymer processing additive containing a multimodal fluoropolymer and melt processable thermoplastic polymer composition employing the same.
Dillon, Maria P., St. Paul, MN, United States
Woods, Susan S., Shoreview, MN, United States
Fronek, Kirsten J., Woodbury, MN, United States
Lavallee, Claude, London, Canada
Amos, Stephen E., Minneapolis, MN, United States
Weilandt, Karl-Dieter, Minneapolis, MN, United States
Kaspar, Harald, Burgkirchen, Germany, Federal Republic of
Hirsch, Bernhard, Burgkirchen, Germany, Federal Republic of
Hintzer, Klaus, Woodbury, MN, United States
Scott, Peter J., Woodbury, MN, United States
Dyneon LLC, Oakdale, MN, United States (U.S. corporation)
US 6277919 B1 20010821
APPLICATION: US 1999-311107 19990513 (9)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 3 OF 5 USPATFULL on STN
1998:135092 Extrudable thermoplastic hydrocarbon compositions.
Blong, Thomas J., Woodbury, MN, United States
Greuel, Michael P., White Bear Township, MN, United States
Lavallee, Claude, London, Canada
Minnesota Mining and Manufacturing, St. Pual, MN, United States (U.S. corporation)
US 5830947 19981103
APPLICATION: US 1997-956090 19971023 (8)
PRIORITY: US 1995-3811P 19950915 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 4 OF 5 USPATFULL on STN
1998:7135 Extrudable thermoplastic hydrocarbon compositions.
Blong, Thomas J., Woodbury, MN, United States
Greuel, Michael P., White Bear Township, MN, United States
Lavallee, Claude, London, Canada
Minnesota Mining and Manufacturing Company, St. Paul, MN, United States (U.S. corporation)
US 5710217 19980120
APPLICATION: US 1995-559789 19951115 (8)
PRIORITY: US 1995-3811P 19950915 (60)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L28 ANSWER 5 OF 5 USPATFULL on STN
96:53373 Melt-processable fluoroplastic.
Blong, Thomas J., Woodbury, MN, United States
Lavallee, Claude, London, Canada
Minnesota Mining and Manufacturing Company, St. Paul, MN, United States

(U.S. corporation)
US 5527858 19960618
APPLICATION: US 1994-300310 19940902 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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ALL L# QUERIES AND ANSWER SETS ARE DELETED AT LOGOFF
LOGOFF? (Y)/N/HOLD:y
STN INTERNATIONAL LOGOFF AT 10:23:13 ON 12 JUL 2004